7-14-2016 12:00 AM

Cholinergic Mechanisms Regulating Cognitive Function and RNA Metabolism

Benjamin Kolisnyk, *The University of Western Ontario*

Supervisor: Drs. Vania, *The University of Western Ontario*
Joint Supervisor: Marco Prado, *The University of Western Ontario*
A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Neuroscience

Abstract

Acetylcholine (ACh) is one of the main neuromodulators in the mammalian central nervous system (CNS). This chemical messenger has been implicated in the underlying physiology of many distinct cognitive functions. However, the exact role that ACh plays in regulating information processing in the brain is still not fully understood. The vesicular acetylcholine transporter (VAChT) is required for the storage of ACh into synaptic vesicles, and therefore it presents a means to modulate release. Diminished VAChT levels cause a decrease in cholinergic tone, whereas increased VAChT expression has been shown to augment ACh release. Previously published data have shown that elimination of VAChT in the basal forebrain in genetically-modified mice impairs learning and memory.

For our studies we have used different mouse lines in which the expression of the VAChT gene is changed, both increased and decreased. We are therefore able to study the consequences of altered cholinergic tone *in vivo*. Our hypothesis is that changes in cholinergic tone produce specific molecular signatures in target brain areas that underlie alterations in cognitive function. Our studies aimed to elucidate the behavioural and molecular consequences of cholinergic dysfunction. Behavioral testing included classical learning and memory tests as well as sophisticated tasks using novel touch screens chambers to measure attention, learning and memory as well as cognitive flexibility. At the molecular level, the goal was to examine how long-term changes in cholinergic tone impact mechanisms regulating synaptic plasticity and neuronal health. Finally, by aging mouse models of cholinergic dysfunction we were able to elucidate the role that cholinergic tone plays in the classical pathological hallmarks of neurodegenerative disorders.

Ultimately, by establishing the molecular signature of increased and decreased cholinergic tone in targeted brain regions (cortex and hippocampus) it may become possible to find novel targets for therapeutic interventions to improve cognitive deficits due to altered cholinergic tone.

Keywords

Acetylcholine

Cholinergic signaling

Vesicular acetylcholine transporter

Cognition

Alzheimer’s Disease

RNA Metabolism

Co-Authorship Statement

Data presented in Chapter 2 of this thesis was previously published in The Journal of Neuroscience on June 19th, 2013 under 33(25)10427-10438. This peer reviewed article is titled “ChAT–ChR2–EYFP mice have enhanced motor endurance but show deficits in attention and several additional cognitive domains”. All of the experiments in Chapter 2, except Figure 2.1A-E, were performed by Benjamin Kolisnyk under the supervision of Dr. Vania F. Prado and Marco A. M. Prado. The confocal microscopy experiments were performed by Dr. Ana Magalhães. The PCR experiments were performed by Jue Fan, and the western blot experiments were performed by Sanda Raulic (University of Western Ontario, London, Canada).

Chapter 3 of this thesis was previously published in The Journal of Neuroscience on September 11th, 2013 under 33(37)14908-14920. This peer reviewed article is titled “Forebrain deletion of the vesicular acetylcholine transporter results in deficits in executive function, metabolic, and RNA splicing abnormalities in the prefrontal cortex”. All of the experiments in Chapter 3, except Figure 3.9, were performed by Benjamin Kolisnyk under the supervision of Dr. Vania F. Prado and Marco A. M. Prado. In vivo magnetic resonance spectroscopy of neuronal metabolites were performed by Dr. Simona Nikolova (University of Western Ontario, London, Canada).

Chapter 4 of this thesis was previously published in the British Journal of Pharmacology on October 1st, 2015 under 170(20)4919-4931. This peer reviewed article is titled “α7 nicotinic ACh receptor-deficient mice exhibit sustained attention impairments that are reversed by β2 nicotinic ACh receptor activation”. All of the experiments in Chapter 4 were performed by Benjamin Kolisnyk with the assistance of Mohamed A. Al-Onaizi under the supervision of Dr. Vania F. Prado and Marco A. M. Prado.

All of the experiments presented in Chapter 5, except for figure 5.1.1B-C,5.1.3 5.2.1I, 5.3A-B, 5.4.1A-F,H,I, 5.4.2, 5.5.1F, 5.6A were performed by Benjamin Kolisnyk under the supervision of Dr. Vania F. Prado and Marco A. M. Prado. RNA-Seq heat map generation and post-hoc analysis of alternative splicing was performed by Dr. Lilach

Soreq (University College London, United Kingdom). Confocal microscopy, silver staining experiments and Morris Water Maze tests and analysis were carried out by Mohamed A. Al-Onaizi under the supervision of Dr. Vania F. Prado and Marco A. M. Prado (University of Western Ontario, London, Canada). The cumulative distribution function analysis of cholinergic gene expression in the AD brain, as well as the mircoRNA sequencing was performed by Shahar Barbash and Uryia Beckenstein under the supervision of Dr. Hemrona Soreq (Hebrew University of Jerusalem, Israel).

All of the experiments presented in Chapter 6, except for figure 6.2A-E, 6.5A were performed by Benjamin Kolisnyk under the supervision of Dr. Vania F. Prado and Marco A. M. Prado. Confocal microscopy was performed by Mohamed A. Al-Onaizi under the supervision of Dr. Vania F. Prado and Marco A. M. Prado (University of Western Ontario, London, Canada).

All other experiments presented in this thesis were performed by Benjamin Kolisnyk under the supervision of Dr. Vania F. Prado and Dr. Marco A. M. Prado at the University of Western Ontario in the Graduate Program in Neuroscience.

Acknowledgments

I would like to acknowledge my family, friends and my cat Cooper. Thank you for your support throughout graduate school. I would also like to thank all present and past members of the Prado lab. Most importantly, I would like to acknowledge Drs. Vania and Marco Prado who provided invaluable insight and mentorship during my doctoral studies.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's Disease
APP	Amyloid Precursor Protein
ChAT	Choline Acetyltransferase
CHT-1	High affinity choline transporter 1
CNS	Central Nervous System
CTL1	*Torpedo*-like choline transporter
GSK-3	Glycogen-synthase kinase-3
hnRNP	Heterogeneous nuclear ribonucleoproteins
LTD	Long term depression
LTP	Long term potentiation
mAChR	Muscarinic acetylcholine receptors
MS	Medial Septum
MSNs	Medium Spiny Neurons
nAChR	Nicotinic acetylcholine receptors
nBm	Nucleus Basalis Magnocellularis
NFT	Neurofibrillary tangles
OCT2	Organic Cation Transporter 2
PFC	Prefrontal Cortex

PNS	Peripheral Nervous System
PPT	Pedunculopontine Tegmentum
RNA-Seq	RNA Sequencing
SR	Serine/Arginine-Rich Protein
STP	Short term potentiation
VAChT	Vesicular Acetylcholine Transporter
VGLUT-3	Vesicular glutamate transporter-3
VP	Ventral Pallidum

Chapter 1

1 Introduction

1.1 Central Cholinergic Tone

Acetylcholine (ACh) was the first neurotransmitter to be identified (Loewi 1921). Cells which secrete, and thus signal via ACh, are deemed cholinergic cells. Cholinergic cells are found throughout the mammalian body and they are both neuronal and non-neuronal cell types. Neuronal ACh acts both in the peripheral nervous system (PNS) and in the central nervous system (CNS). In the PNS ACh is the neurotransmitter which activates skeletal muscles, in the neuromuscular junction. Furthermore, ACh in the PNS is the neurotransmitter of the autonomic nervous system which regulates a host of involuntary and unconscious bodily functions. CNS ACh is a neuromodulator affecting synaptic plasticity and regulating many behaviours including learning and memory, attention, and reward (Hasselmo 1999, Picciotto, Higley et al. 2012).

1.1.1Regulation of ACh Release

Synthesis of ACh relies on the uptake of choline into the presynaptic cholinergic nerve terminal. This process is mediated by the activity of the high affinity choline transporter 1 (CHT-1) (Yamamura and Snyder 1972) Kuhar and Murrin 1978). Choline in the presynaptic cell is then combined with Acetyl-CoA and synthesized into ACh by the choline acetyltransferase enzyme (ChAT) (Hersh 1982, Rylett and Schmidt 1993). Newly synthesized molecules of ACh are then packaged into synaptic vesicles by the vesicular acetylcholine transporter (VAChT) (Prado, Reis et al. 2002, Prado, Roy et al. 2013). VAChT represents the rate liming step in the release of ACh. Genetic elimination of VAChT has demonstrated that VAChT is required for the storage and release of ACh (Prado, Martins-Silva et al. 2006, de Castro, De Jaeger et al. 2009). Unlike monoamine which have multiple and redundant transporters (Liu, Peter et al. 1992), VAChT is the sole transporter for ACh. Following stimulation of the presynaptic cell, ACh containing

synaptic vesicles fuse with the presynaptic membrane and ACh is released into the synapse (Katz and Miledi 1965, Varoqui and Erickson 1996). In the synapse, ACh can bind and act upon pre and post synaptic ACh receptors. These receptors fall into two main classes, ionotropic nicotinic receptors, and metabotropic muscarinic receptors. Upon dissociation from the receptors, synaptic ACh is broken down into its constituents by the activity of the acetylcholinesterase enzyme (AChE) (Marnay and Nachmansohn 1937, Soreq and Seidman 2001). Schematic of ACh release is depicted in Figure 1.1.

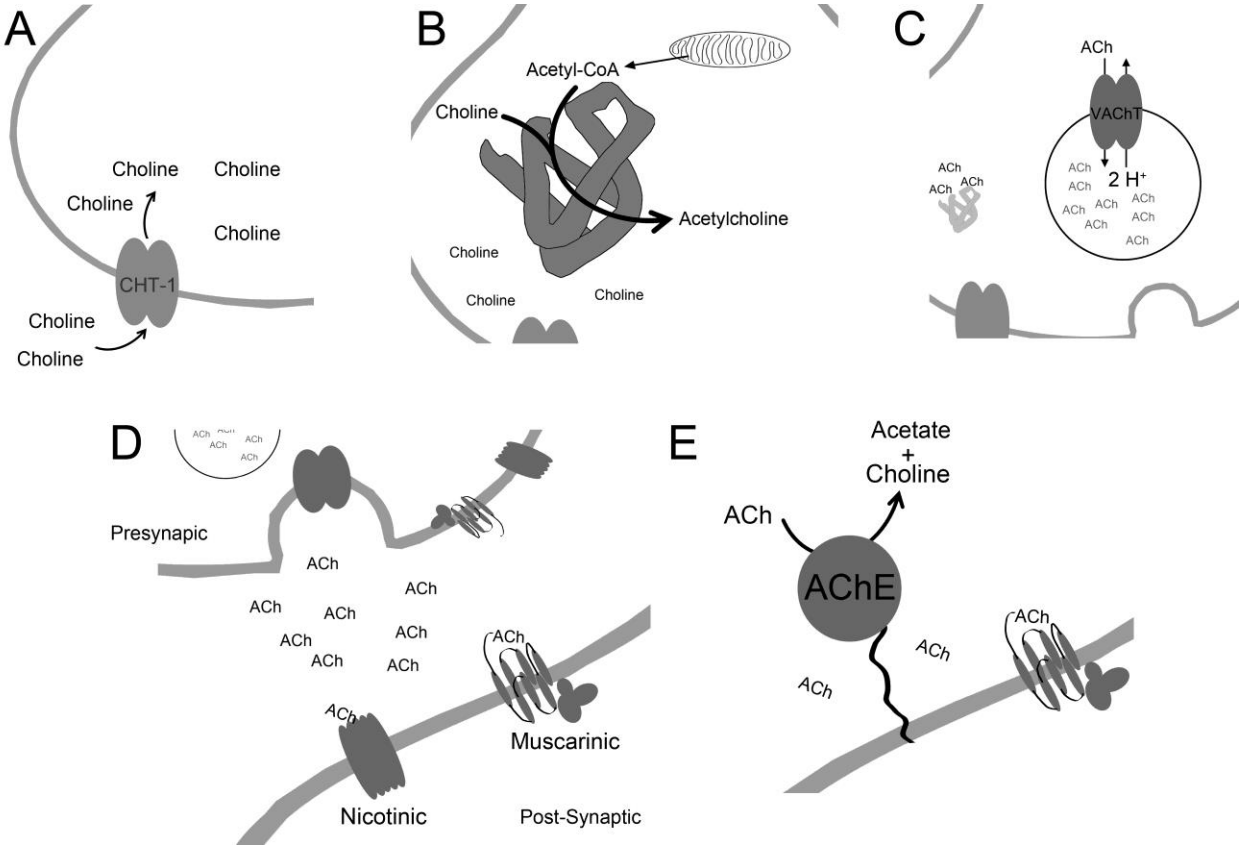


Figure 1.1 The Cholinergic Synapse. (A) Choline is taken up into the pre-synaptic cholinergic neuron by the activity of the high affinity choline transporter, CHT-1. (B) Choline and acetyl-CoA are combined to form acetylcholine by the choline acetyltransferase enzyme. (C) The Vesicular Acetylcholine Transporter packages acetylcholine into synaptic vesicles, exchanging one cytoplasmic molecule of acetylcholine for two vesicular H^+ ions. (D) Following excitation of the pre-synaptic cholinergic neurons, acetylcholine containing synaptic vesicle fuses with the plasma membrane and acetylcholine is released into the synapse. Acetylcholine can then bind to either ionotropic nicotinic receptors or metabotropic muscarinic receptors. These receptors can be expressed both pre- and post-synaptically. (E) Following dissociation of acetylcholine from its receptors, the molecule is broken down by the acetylcholinesterase enzyme into acetate and choline.

1.1.2The Vesicular Acetylcholine Transporter

To speed up synaptic transmission, neurotransmitters are packaged into synaptic vesicles, and released as a quantum (Katz and Thesleff 1957, Stevens 1993). This quantal release mechanism allows for the local concentration of neurotransmitters released into the synapse to be orders of magnitude larger than if neurotransmitters were simply released uniformly across the nerve terminal. Release events of ACh from neurons into the synapse have been well characterized as quantal events (Katz and Thesleff 1957, Van der Kloot and Molgo 1994). It has been estimated that roughly 10,000 molecules of ACh are packaged into each vesicle (Linder, Pennefather et al. 1984). As ACh is a cation, it will not simply diffuse passively through membranes. A transport mechanism is required for the packaging of ACh into vesicles for synaptic release. VAChT is the protein responsible for the packaging of ACh into synaptic vesicles (Alfonso, Grundahl et al. 1993). Evidence of how VAChT carries out this transport has been elucidated from models of the proteins structure.

Although the structure of the protein has not been resolved by crystallography or other methods, models of the protein structure have been proposed to explain the biochemical activity of the transporter. It has been suggested, based on the knowledge of other transporters in the same protein family, that VAChT would assume a 12 transmembrane domain structure (Vardy, Arkin et al. 2004). Following the proposed model, the protein would be separated into two distinct large structural halves, allowing for a central transport path to be formed between them (Khare, Ojeda et al. 2010). VAChT is able to pump ACh into synaptic vesicles, by exchanging two intra-vesicular protons, for one cytoplasmic molecule of ACh (Nguyen, Cox et al. 1998). Experimental evidence suggests that this exchange occurs within the described central transport path of the protein (Ojeda, Kolmakova et al. 2004).

In vitro studies have indicated that the activity of VAChT is highly regulated. VAChT will concentrate ACh into vesicles at a rate 30 folds lower than predicted, based on the free energy formed by the exchange of two protons (Parsons 2000). The pharmacological agent vesamicol is a selective non-competitive VAChT inhibitor. Studies using

vesamicol have shown that the compound is able to block ACh storage in vesicles, and also block release of ACh into the synapse (Anderson, King et al. 1983, Collier, Welner et al. 1986, Whitton, Marshall et al. 1986, Prado, Gomez et al. 1992, Van der Kloot 2003). Therefore, synaptic release of ACh is dependent on VAChT packaging the neurotransmitter into vesicles. These findings have been confirmed *in vivo*, where mice with targeted deletions of VAChT no longer release ACh upon KCl stimulation (Prado, Martins-Silva et al. 2006, de Castro, De Jaeger et al. 2009, Lima Rde, Prado et al. 2010, Guzman, De Jaeger et al. 2011). Taken together, these results place VAChT as the rate limiting step in the release of ACh into the synapse.

The majority of total VAChT protein is found within the synaptic vesicles of cholinergic neurons. The VAChT protein however is not locally translated at the synapse (Park, Gondre-Lewis et al. 2011). The intracellular trafficking of VAChT is therefore a key cellular process within cholinergic neurons (Prado and Prado 2002). VAChT trafficking to synaptic vesicles has been shown to be a clathrin-mediated process (Santos, Barbosa et al. 2001, Barbosa, Ferreira et al. 2002, Ferreira, Santos et al. 2005). Experiments using a yeast two hybrid system to investigate protein-protein interaction, have shown that the C-terminal domain of VAChT interacts with the AP-2 adaptor complex (Barbosa, Ferreira et al. 2002). AP-2 is a multimeric protein that is a master regulator of chlatrin-mediated endocytosis (Pearse, Smith et al. 2000). A di-leucine motif has been characterized within the C-terminus of the VAChT protein, and plays a critical role in the clathrin-mediated endocytosis of the transporter (Tan, Waites et al. 1998). Mutations in this di-leucine motif abolish the interaction between VAChT and the AP-2 complex (Barbosa, Ferreira et al. 2002). Likewise, the C-terminal segment of vesicular monoamine transporters (VMATs) regulates their trafficking (Tan, Waites et al. 1998). This is not surprising as there is a large degree of homology between VAChT and VMATs (Liu and Edwards 1997). Interestingly, the intracellular trafficking of these proteins is vastly different. In the brain, VMATs are trafficked to many different types of secretory vesicles (Nirenberg, Liu et al. 1995, Nirenberg, Chan et al. 1996). Conversely, VAChT is localized almost exclusively to synaptic vesicles (Gilmor, Nash et al. 1996, Weihe, Tao-Cheng et al. 1996). It appears that the phosphorylation of this C-terminus is important to the subcellular specificity of VAChT trafficking (Krantz, Waites et al. 2000,

Ferreira, Santos et al. 2005). The kinase PKC has been shown to both modulate VAChT phosphorylation as well as ACh release from hippocampal synaptosomes (Tanaka, Fujiwara et al. 1986, Allgaier, Daschmann et al. 1988, Barbosa, Clarizia et al. 1997). VAChT phosphorylation can therefore be highly relevant to cholinergic signalling in the brain (Van der Kloot and Molgo 1994).

1.1.3 Choline Acetyltransferase

Synthesis of ACh within neurons occurs within the cytoplasm of cholinergic nerve terminals. ChAT is the enzyme responsible for the production of ACh within cells. As its name suggests, choline acetyltransferase transfers an acetyl group from an acetyl-CoA to a choline molecule to produce the neurotransmitter ACh (Nachmansohn and Berman 1946). Expression of the ChAT enzyme is extremely sparse, estimated to comprise less than a ten thousandth of a percent of total brain proteins (Eckenstein and Thoenen 1982, Bruce, Wainer et al. 1985). Despite this low abundance, ChAT protein is found within every cholinergic cell in the brain (Docherty, Bradford et al. 1985), its expression pattern therefore follows the anatomical distribution of cholinergic neurons in the brain.

Interestingly, the genes for ChAT and VAChT are present in the same gene locus, termed the cholinergic gene locus (Eiden 1998, Mallet, Houhou et al. 1998). Cloning of the ChAT gene revealed a rather unique structure for the gene (Berrard, Brice et al. 1987, Strauss, Kemper et al. 1991). Upstream of the coding region for ChAT, within the intron between the first and second exons, the VAChT 1590bp open reading frame was discovered (Bejanin, Cervini et al. 1994, Erickson, Varoqui et al. 1994, Roghani, Feldman et al. 1994). The VAChT gene is found in the same transcriptional orientation as ChAT (Berrard, Varoqui et al. 1995). This unique gene organization is evolutionarily conserved and can be found in *C. elegans* (Alfonso, Grundahl et al. 1994, Roghani, Feldman et al. 1994), drosophila (Kitamoto, Wang et al. 1998), mice (Misawa, Ishii et al. 1992, Barbosa, Massensini et al. 1999), rats (Bejanin, Habert et al. 1992) and men (Berrard, Brice et al. 1987). Although arising from the same gene locus, VAChT and

ChAT are transcribed as separate transcripts (Alfonso, Grundahl et al. 1994, Erickson, Varoqui et al. 1994).

ChAT is localized predominantly in the cytoplasm (Bruce, Wainer et al. 1985). However, membrane-bound forms of the protein have been reported (Bruce and Hersh 1987). In addition, a variant of the human form of the enzyme can localize to the nucleus, wherein it plays a role in the regulation of gene expression and chromatin modeling (Gill, Bhattacharya et al. 2003, Matsuo, Bellier et al. 2011). The ChAT enzyme is organized as a globular, single strand, protein (Govindasamy, Pedersen et al. 2004). Site-directed mutagenesis studies have shown that a number of critical amino acid residues regulate the catalytic function of the protein (Carbini and Hersh 1993, Dobransky, Davis et al. 2000). This work showed that a histidine residue is the acid/base catalytic residue of the enzyme, while a nearby arginine residue interacts with, and binds the CoA molecule. Further structural biology experiments have confirmed these findings (Kim, Rylett et al. 2006).

Interestingly, there appears to be no correlation between the mRNA level of ChAT within a cellular population, and the enzymatic activity of the cells (Berrard, Brice et al. 1987). This finding suggests that regulation of ChAT protein levels and activity occur at the post-transcriptional level. Translation of the ChAT protein occurs within the soma of neurons (Berrard, Brice et al. 1987) and the enzyme is then transported by an undetermined mechanism to the synapse. The transport of the enzyme represents a means by which cells can regulate their production of ACh. A critical post-translational modification to the ChAT enzyme is its phosphorylation (Bruce and Hersh 1989, Dobransky and Rylett 2003). A number of phosphorylation sites have been demonstrated on the enzyme and it appears that these modifications play a role in regulating the catalytic activity of the enzyme (Dobransky and Rylett 2003). A key phosphatase involved in the regulation of ChAT enzymatic activity is PKC (Dobransky, Doherty-Kirby et al. 2004). Importantly ChAT phosphorylation in synaptosomes is sensitive to Ca^{2+} levels (Dobransky and Rylett 2005). The enzymatic activity of ChAT, and consequently ACh synthesis, can therefore be regulated in response to neuronal depolarization. Deletion of the chat gene from the mouse genome is post-embryonically

lethal (Misgeld, Burgess et al. 2002). Studies of the embryos from these animals have shown in the absence of ChAT there is dramatic restructuring of the PNS in these animals (Brandon, Lin et al. 2003), indicating that ChAT, and therefore ACh, is essential for proper developments of the nervous system.

1.1.4 The High Affinity Choline Transporter

Synthesis of ACh is dependent on the intracellular concentrations of choline. Neurons are unable to synthesise enough of their own choline for cholinergic neurotransmission (Yamamura and Snyder 1972, Collier and Katz 1974).Therefore, ACh releasing neurons are dependent on the activity of a transporter to obtain their choline (Birks, Macintosh et al. 1956, Birks and Macintosh 1957). The transporter responsible for the uptake of choline into neurons is the CHT1 transporter. This transporter regulates choline levels within a cell, by transporting choline into the cell in a Na^+ dependant manner (Simon and Kuhar 1975, Birks, Worsley et al. 1985). This protein is encoded by the SLC5A7 gene, which was first identified in *C. elegans*, and subsequently identified in the rat, mouse and human (Apparsundaram, Ferguson et al. 2000, Okuda, Haga et al. 2000). There is a very high degree of homology between the rat and human gene (Okuda and Haga 2000) as well as between the rat and mouse gene (Apparsundaram, Ferguson et al. 2001). Importantly, CHT1 is preferentially expressed in cholinergic neurons, and can therefore be used as a specific marker for these cells (Misawa, Nakata et al. 2001).

CHT1 is not the sole choline transporter expressed in the mammalian brain. The *Torpedo*-like choline transporter, CTL1 is highly expressed in oligodendrocytes, where it provides choline for the synthesis of phospholipids (Traiffort, Ruat et al. 2005). CLT1 also differs from CHT1 in that its function is dependent on K^+ ions rather than Na^+ ions (Fujita, Shimada et al. 2006). The other choline transporter expressed in the mammalian brain is the organic cation transporter 2 (OCT2). Unlike CTL1, OCT2 is also expressed in cholinergic neurons, being found on synaptic vesicles within these neurons (Nakanishi, Haruta et al. 2011, Nakata, Matsui et al. 2013). OCT2 however is not selective for choline, but rather can transport all organic cations. Given that OCT2 and CHT1 share similar localization in cholinergic neurons, it has been hypothesized

that these two transporters may collaborate to transport choline into cholinergic neurons (Nakata, Matsui et al. 2013).

The CHT1 protein is 580 amino acids long and organized on plasma membranes in a 13 transmembrane domain structure (Apparsundaram, Ferguson et al. 2000, Okuda, Haga et al. 2000, Torres, Gainetdinov et al. 2003). CHT1 has been shown to have consensus PKA and PKC phosphorylation motifs, implicating these signalling pathways in the regulation of this protein (Gates, Ferguson et al. 2004, Brock, Nickel et al. 2007). Experimental inhibition of these signalling pathways has led to decreases in cell surface levels of the transporter (Gates, Ferguson et al. 2004). Although CHT1 carries out its physiological function at the plasma membrane, the vast majority of the protein is found within intracellular vesicles, with only a small portion of the total protein pool found on the plasma membrane (Ferguson, Savchenko et al. 2003, Ribeiro, Alves-Silva et al. 2003, Ribeiro, Black et al. 2005). Inclusion of CHT1 in the plasma membrane of synapses is dependent on the activity of the neuron. Depolarization by action potential of the neuron increases the levels of CHT1 at the plasma membrane in a calcium dependant manner (Collier and Katz 1974, Simon and Kuhar 1975). The trafficking of CHT1 to the plasma membrane is mediated by the clathrin endocytic pathway (Ribeiro, Alves-Silva et al. 2003). Plasma membrane CHT1 then can be internalized in a clathrin dependant manner to either be tagged for proteasome degradation, or to be trafficked back to the plasma membrane (Ribeiro, Black et al. 2005).

Genetic elimination of CHT1 from the mouse genome results in post-natal lethality, within an hour of birth, due to an inability of the animals to breathe (Ferguson, Bazalakova et al. 2004). Mice with a heterozygous null mutation are however viable and are able to maintain the same level of choline uptake as wild-type control animals through posttranslational compensation of CHT1 function, by increased inclusion at the plasma membrane (Ferguson, Bazalakova et al. 2004). Furthermore, in mice with a heterozygous null deletion of the ChAT gene (and therefore a reduced capacity to synthesize ACh), levels of the CHT1 protein were increased in compensation (Brandon, Mellott et al. 2004). Analysis of the prefrontal cortex of rats performing attention based cognitive tasks showed both an increase in choline uptake and in CHT1 protein levels

(Apparsundaram, Martinez et al. 2005). These findings demonstrate that the trafficking of CHT1 to the plasma membrane is a highly dynamic process which plays a role in normal physiological functioning. Importantly polymorphisms in the CHT-1 gene have been identified in patients with Attention Deficit Disorders, related to cholinergic deficits in these patients (English, Hahn et al. 2009). The trafficking of CHT1 to the plasma membrane is thus a critical determinant to the levels of choline within cholinergic cells and a critical process for cognitive functioning.

1.1.5 Nicotinic Receptors

Nicotinic acetylcholine receptors (nAChRs) are ionotropic receptors which are selective for ACh. nAChRs are expressed in both the peripheral and in the CNS. This class of receptors are involved in neuronal excitability and regulation of neurotransmitter release. There are nine different nicotinic receptor subunits that are expressed in the CNS, they are either α subunits (α2, 3, 4, 5, 6, 7) or β subunits (β2, 3, 4) all encoded by distinct genes (Le Novere and Changeux 1995, Dani and Bertrand 2007). These subunits combine as either homomeric or heteromeric pentameric receptors. The most prominent homomeric nicotinic receptor in the mammalian CNS is the α7 nicotinic receptor (α7nAChR). However the most prominently expressed nicotinic receptor in the brain is the α4β2nAChR (Wada, Wada et al. 1989).

Regardless of their make-up, nicotinic receptors share the same general structure. They are organised as a transmembrane receptor with a central ion channel, an extracellular ligand binding domain with a ligand binding pocket (Karlin and Akabas 1995). Although they share similar structures, α7nAChRs and α4β2nAChRs have drastically different functional properties (Giniatullin, Nistri et al. 2005). α7nAChRs are quick to be activated by ACh and are quickly desensitized (Pidoplichko, DeBiasi et al. 1997). These receptors are permeable to Na^+, and K^+, but are highly permeable to Ca^{2+} (Seguela, Wadiche et al. 1993). α4β2nAChRs on the other hand are slow to be activated by ACh and are also slow to desensitize (Alkondon and Albuquerque 2005). These receptors are only permeable to Na^+ and K^+ ions.

In situ hybridization studies have demonstrated that the α4 and β2 nAChR subunits are expressed ubiquitously across the mammalian brain (Wevers, Jeske et al. 1994). Expression of these receptors is highest in the thalamus and cortex and relatively low in the hippocampus (Alkondon, Reinhardt et al. 1994). β2 nAChRs is essential for nicotine evoked release of GABA and dopamine from synaptosomes collected from a number of brain regions (Turner 2004, McClure-Begley, King et al. 2009). Furthermore nicotine evoked striatal release of dopamine is abolished in mice lacking the β2 subunit (King, Caldarone et al. 2004). This regulation of dopamine release by β2nAChRs is functionally relevant as mice lacking this receptor do not display place preference conditioning to cocaine (Zachariou, Caldarone et al. 2001). Young mice lacking the β2 nAChR subunit do not display memory impairments, however as these mice age they display cortical atrophy and cell loss in the hippocampus (Zoli, Picciotto et al. 1999). The age dependant neurodegeneration in these animals is associated with learning and memory impairments.

The α7nAChR like the α4 and β2 is expressed throughout the brain. However the distribution pattern of the α7nAChR is antithetical to the pattern of the α4 and β2 subunits. α7nAChRs are most expressed in the hippocampus and cortex, but are almost absent in the thalamus (Seguela, Wadiche et al. 1993). Although α7nAChRs are highly involved in synaptic plasticity in the hippocampus, α7-subunit knockout mice did not differ from wild-type controls in a host of memory assays including contextual and cued fear-conditioning and on the Morris water maze (Paylor, Nguyen et al. 1998, Fernandes, Hoyle et al. 2006). α7nAChRs are also expressed on non-neuronal cells in both the brain and in the periphery, including astrocytes and microglia. α7nAChRs on these non-neuronal cells play a critical role in inflammation (de Jonge and Ulloa 2007) and in neuroprotection (Ren, Puig et al. 2005). On immune cells activation of α7 receptors regulates the production of inflammatory cytokines (Wang, Yu et al. 2003).

1.1.6 Muscarinic Acetylcholine Receptors

ACh can act upon a class of metabotropic receptors, the muscarinic acetylcholine receptors (mAChRs). There have been 5 subtypes of mAChRs that have been

identified, the M1, M2, M3, M4 and M5 receptors. All of these receptors function as classical G-protein coupled receptors (Wess 1996). mAChRs can be subdivided into two categories, the Gq coupled receptors which includes the M1, M3 and M5 receptors (Berstein, Blank et al. 1992, Offermanns, Wieland et al. 1994, Qin, Dong et al. 2011), and the Gi/o coupled M2 and M4 receptors (Winitz, Russell et al. 1993, Migeon and Nathanson 1994). The Gq coupled receptors activate phospholipase C and increase intracellular Ca^{2+} levels; these receptors are therefore deemed excitatory. The Gi/o coupled receptors on the other hand act by decreasing cyclic nucleotide levels, decreasing intracellular Ca^{2+} levels and promoting K^{+} efflux, thus inhibiting the neuron. G proteins can also directly regulate K^{+} channels, which is a common mechanism for M2 muscarinic receptors (Kunkel and Peralta 1995).

All five of the mAChR are expressed in the mammalian nervous system. These receptors are found to be expressed on both neuronal and glial cell types in the nervous system. The expression of the M1, M4 and M5 subtypes is enriched in the CNS, whereas the M2 and M3 receptors are equally found in both the CNS and the PNS (Levey 1993). mAChRs in the brain have been shown to play regulatory roles in many cognitive processes. Each of the five muscarinic receptors are encoded by a unique gene. This has allowed for the genetic manipulation of the individual receptor types and from this their distinct physiological roles in the central nervous system have been elucidated (Wess 2004).

M1 mAChRs are expressed throughout the mammalian forebrain, including cerebral cortex, hippocampus, and in the striatum (Wolfe and Yasuda 1995). It is by its broad level of expression that the receptor is thought to play a role in many cognitive processes. Mice lacking the M1 mAChR have been shown to be hyperactive (Gerber, Sotnikova et al. 2001) and have impaired working memory (Anagnostaras, Murphy et al. 2003) and consolidation (Gould, Dencker et al. 2015). These animals also present a number of biochemical (Berkeley, Gomeza et al. 2001) and electrophysiological (Anagnostaras, Murphy et al. 2003) abnormalities that underlie their impaired behaviour.

M3 mAChRs are expressed throughout the brain but are most prominently expressed in the hypothalamus (Wall, Yasuda et al. 1991). Hypothalamic M3 mAChRs regulate food intake and appetite. These receptors are found on melanin-concentrating hormone producing neurons of the hypothalamus, and their activations stimulates production of the appetite regulating hormones (Yamada, Miyakawa et al. 2001). Mice lacking this receptor are lean and have a pronounced reduction in bodyweight and food intake compared to control animals (Yamada, Miyakawa et al. 2001). In the periphery these receptors have been shown to regulate smooth muscle function (Matsui, Motomura et al. 2002) and the salivary response (Bacman, Sterin-Borda et al. 1996).

M5 mAChRs have low levels of expression throughout the brain and are enriched in limited cellular populations (Wei, Walton et al. 1994). M5 mAChRs are expressed on the midbrain dopaminergic neurons, originating from the substantia nigra (Vilaro, Palacios et al. 1990), and are therefore highly critical to the regulation of dopamine release in the striatum. M5 mAChRs have been shown to directly regulate striatal dopamine release, although other muscarinic subtypes can also modulate striatal dopamine by indirect mechanisms (Zhang, Yamada et al. 2002). The ventral tegmental area of mice deficient in the M5 mAChR do not respond to cholinergic stimulation demonstrating that these receptors also regulate ventral tegmental area dopamine release (Yeomans, Forster et al. 2001, Forster, Yeomans et al. 2002). Given the role of these receptors in regulating dopamine signalling, mice lacking M5 mAChRs have altered responses to opioids and cocaine (Basile, Fedorova et al. 2002, Thomsen, Woldbye et al. 2005).

The inhibitory M2 and M4 mAChRs function mainly as auto-receptors for ACh in the brain (Starke, Gothert et al. 1989). These receptors are also both present on the GABAergic medium spiny neurons in the striatum (Yan, Flores-Hernandez et al. 2001). For the most part, these receptors are responsible for inhibiting release of ACh (Zhou, Meyer et al. 2002). They are therefore predominantly presynaptic receptors found on

cholinergic neurons (Levey, Edmunds et al. 1995). These receptors therefore play a critical role in the regulation of the timing and release of ACh, processes which are highly relevant to cognitive function (Sarter, Parikh et al. 2009). Mice lacking the M2 receptor show a broad range of hippocampal behavioural and electrophysiological impairments (Seeger, Fedorova et al. 2004). Mice lacking the M4 receptor also present with learning impairments and hyperactivity (Koshimizu, Leiter et al. 2012). Given that these receptors regulate release of ACh; it is difficult to differentiate the specific roles of these receptors to cognition, from general roles of cholinergic tone in the brain.

1.1.7 Basal Forebrain Cholinergic System

The basal forebrain is a collection of nuclei including the medial septum (MS), the ventral pallidum (VP), the diagonal band nuclei, magnocellular preoptic nucleus, and the nucleus basalis magnocellularis (nBm) (Zaborszky, Pang et al. 1999). The neurons found within this brain region have been implicated in many cognitive functions including learning and memory and executive function (Miyamoto, Shintani et al. 1985). The basal forebrain is composed of a highly variable population of neurons. ACh releasing neurons represent roughly 20% of the neurons in the basal forebrain (Semba 2004). Other neurons in the basal forebrain release glutamate, GABA and neuropeptides (Henny and Jones 2008).

The output of basal forebrain cholinergic neurons is dependent on the nuclei from which these neurons are found (Figure 1.2A). Neurons originating in the MS and the diagonal band nuclei provide the major cholinergic innervation to the hippocampus (Mesulam, Mufson et al. 1983). Cholinergic neurons from the diagonal band and from the magnocellular preoptic nucleus project to the olfactory bulb and to the enthorinal cortex (Gaykema, Luiten et al. 1990), while the nBm cholinergic neurons project to both the basolateral amygdala and the entire cortex (Boegman, Cockhill et al. 1992, Power, Thal et al. 2002). Just as the basal forebrain is not a homogenous cell population, the described projections are not purely cholinergic with both GABAergic and glutamatergic projection neurons sharing the same projection tracks as the cholinergic cells (Huh, Goutagny et al. 2010).

Basal forebrain cholinergic neurons receive afferent input from midbrain and brainstem as well as from the hypothalamus. Unlike the non-cholinergic neurons in the basal forebrain, the cholinergic neurons in the basal forebrain do not appear to receive top down input from the cortex (Zaborszky 1989, Zaborszky and Duque 2000). Hypocretin/orexin positive neurons from the hypothalamus have been identified as the main source of innervation to the basal forebrain (Henny and Jones 2006). These neurons have been shown to synapse onto both cholinergic and non-cholinergic neuron in the basal forebrain. These neurons are histaminergic and are important regulators of arousal. Midbrain afferent connections to the basal forebrain originate from the dopaminergic cells of the substantia nigra and from the ventral tegmental area (Fallon and Moore 1978, Zahm and Trimble 2008). Brainstem structures which project to the basal forebrain cholinergic cells are adrenergic in nature from the medulla and the locus coeruleus and cholinergic from the pedunculopontine nucleus.

Basal forebrain cholinergic neurons role in regulating executive function and memory has largely been investigated by immunotoxin lesioning of these cells (Baxter, Bucci et al. 2013). The 192 IgG-saporin is a specific neurotoxin which, when delivered to the basal forebrain, will selectively kill cholinergic neurons and spare the other cell types in the region (Book, Wiley et al. 1992). An important caveat to the attribution of these behaviours to cholinergic signalling is that these neurons may not be simply cholinergic. In culture, these cholinergic neurons from the basal forebrain have been shown to be able to release glutamate (Allen, Abogadie et al. 2006). Recent optogenetic experiments have demonstrated that basal forebrain cholinergic which project to the cortex co-release GABA from distinct pools of vesicles (Saunders, Granger et al. 2015). The behavioural consequences of this co-transmission have yet to be investigated, but suggest that GABA and/or glutamate co-released with ACh may influence behaviours thought to be regulated by ACh signalling alone (Granger, Mulder et al. 2016).

1.1.8 Striatal Cholinergic System

Contrary to projection cholinergic neurons found in the basal forebrain and the pedunculopontine tegmental nucleus, the cholinergic neurons in the striatum are local

interneurons (Figure 1.2B). As such, they are characterized by shorter axons and form circuits with nearby neuron within the same brain region (Zhou, Wilson et al. 2002). The cholinergic interneurons of the striatum are the primary source of ACh in the striatum, but only comprise less than 1% percent of all cells in the striatum (Bolam, Wainer et al. 1984). Cholinergic neurons in the striatum, relative to other cholinergic neurons, have higher levels of the key cholinergic markers choline acetyltransferase (Hebb and Silver 1961) and acetylcholinesterase (Woolf and Butcher 1981). This enrichment in cholinergic markers demonstrates the importance that these cells play in regulating striatal function. 33 1.2 B.

A key role of the cholinergic interneurons in the striatum is to integrate information from afferent inputs to the striatum. This is achieved by assimilating inputs of a large number of neurons which release various different neurotransmitters, by expressing a large number of different receptor classes for these neurotransmitters. These neurotransmitters can either act in an excitatory manner, such as glutamate (Calabresi, Centonze et al. 1998), serotonin (Bonsi, Cuomo et al. 2007), histamine (Bell, Richardson et al. 2000) and substance P (Aosaki and Kawaguchi 1996). Neurotransmitters can also inhibit cholinergic interneurons, such as GABA (DeBoer and Westerink 1994), adenosine (Brown, James et al. 1990), and endogenous opioids (Rada, Mark et al. 1991). Other neurotransmitters can be both excitatory and inhibitory, depending on which class of receptors they activate such as with dopamine, excitatory D1-like signalling (Aosaki, Kiuchi et al. 1998) and inhibitory D2-like (Chuhma, Mingote et al. 2014). It is therefore the role of these cholinergic interneurons to integrate all of these various signals.

Despite their relatively small cell number, striatal interneurons project throughout the striatum and can therefore have broad physiological effects. GABA-ergic medium spiny neurons (MSNs) are the primary output of the striatum (Chuhma, Tanaka et al. 2011). These neurons are not thought to express nicotinic acetylcholine receptors (Luo, Janssen et al. 2013), though nicotine can indirectly modulate their activity (Liu, Otsu et al. 2007). Cholinergic signalling therefore directly modulates the activity of MSNs by

activation of muscarinic receptors. Activation of M1 muscarinic receptors on these cells is thought to reduce KCNQ potassium inhibition (Shen, Hamilton et al. 2005) and Ca^{2+} entry (Perez-Garci, Bargas et al. 2003), increasing the excitation of MSNs. Nicotinic receptors in the striatum are highly concentrated on both glutamatergic and dopaminergic nerve terminals. Glutamatergic nerve terminals in the striatum are enriched in α7 nicotinic receptors, and activation of these receptors leads to release of glutamate (Campos, Alfonso et al. 2010). Release of dopamine in the striatum is also highly dependent on cholinergic signalling. Unlike glutamate, the release of dopamine in the striatum has been shown to be dependent on the synchrony of cholinergic interneuron firing, and the activation of β2 nicotinic receptors on dopaminergic terminals (Cachope, Mateo et al. 2012).

An important caveat for many of the studies of striatal cholinergic neurons, is that these cells co-transmit glutamate. This co-transmission is achieved through synergism between VAChT and the vesicular glutamate transporter-3 (VGLUT-3) (El Mestikawy, Wallen-Mackenzie et al. 2011). VAChT and VGLUT-3 are found on the same vesicles in these neurons and it has been shown that VGLUT-3 enables these vesicles to package additional ACh (Gras, Amilhon et al. 2008). Studies using genetically modified mice have shown that the glutamate and the ACh released from these neurons can have separate and potentially opposite physiological roles in regulating striatal function and behaviour (El Mestikawy, Wallen-Mackenzie et al. 2011, Guzman, De Jaeger et al. 2011, Sakae, Marti et al. 2015). Behavioural studies of mice with lesioned striatal cholinergic interneurons were found to be hyperactive, have a heightened response to cocaine and do not show haloperidol induced catalepsy (Hikida, Kaneko et al. 2001, Kitabatake, Hikida et al. 2003). These behaviours were initially attributed to the cholinergic properties of these cells. However mice lacking release of ACh from the striatum were not hyperactive and did not display heightened responses to cocaine (Guzman, De Jaeger et al. 2011). In fact, it was mice lacking the VGLUT-3 transporter which recapitulated the phenotype of the lesioned animals (Gras, Amilhon et al. 2008). The ACh released from these cells seems to be playing a critical role in regulating release of dopamine. The animals lacking release of ACh from the striatum showed a

functional increase in dopamine receptor expression and activity, assessed by pharmacological MRI (Guzman, De Jaeger et al. 2011). VGLUT-3 and VAChT have opposing roles in the regulation of dopamine release. Elimination of VAChT from striatal interneurons decreases KCl stimulated dopamine release, whereas elimination of VGLUT-3 from these neurons potentiates the dopamine response (Sakae, Marti et al. 2015). These results suggest that other physiological properties of striatal ACh may potentially be attributable to the glutamate released from these cholinergic interneurons or may be dependent on the combined action of both neurotransmitters.

1.1.9 The Pedunculopontine Tegmentum Cholinergic System

The pedunculopontine tegmentum (PPT) is a collection of nuclei found within the brainstem behind the substantia nigra. The nuclei found within the PPT are a heterogeneous population of neurons that differ by the neurotransmitters they release. The three major neurons within the PPT are cholinergic, GABAergic and glutamatergic (Martinez-Gonzalez, Wang et al. 2012). These three types of neurons are largely segregated into separate regions of the PPT. The majority of cholinergic neurons within the PPT are located in the PPT pars compacta (Pahapill and Lozano 2000).

Much of the work to characterize the projection of the cholinergic neurons from the PPT has been done in non-human primates. These experiments have shown that these cholinergic neurons project predominantly to structures within the basal ganglia (Figure 1.2 C). Both the substantia nigra (Futami, Takakusaki et al. 1995) and the ventral tegmental area receive significant input from the cholinergic neurons of the PPT (Charara, Smith et al. 1996). This anatomical data place these cholinergic cells as key regulators of dopamine signalling in the brain. The primary output of the cholinergic neurons from the PPT is the thalamus (Parent, Pare et al. 1988). Additionally the striatum also receives direct input from the cholinergic neurons of the PPT (Dautan, Huerta-Ocampo et al. 2014). The PPT is therefore an alternate source of cholinergic tone in the striatum, besides the cholinergic interneurons. Finally an important target of the cholinergic neurons in the PPT is the contralateral PPT (Benarroch 2013).

Given the anatomical connectivity data, the cholinergic output from the PPT is placed to be a key regulator of dopamine signalling in the brain. Experimental evidence for this was first shown by studying the addictive properties of nicotine; by increasing dopamine release acting specifically through β2 nicotinic acetylcholine receptors in the VTA (Picciotto, Zoli et al. 1998). Further work has also demonstrated that M5-type muscarinic receptors can also stimulate dopamine release from the VTA (Corrigall, Coen et al. 2002). Since the cholinergic output from the PPT can robustly modulate dopamine signaling, these neurons are important intermediaries of addiction and reward. Not only do these neurons release more ACh into the VTA during cocaine administration and cocaine seeking behaviours (You, Wang et al. 2008), but these cholinergic neurons will also increase their firing rate in response to environmental cues associated with reward (Goldberg and Reynolds 2011). Given these findings, cholinergic PPT neurons have been proposed to be master regulators of dopamine signalling in the brain (Maskos 2008).

The PPT cholinergic neurons also play a key role in regulating sleep and arousal. PPT cholinergic neurons are completely inhibited during seizures, this inhibition is accompanied by changes in EEG recordings (Motelow, Li et al. 2015). In line with these findings, PPT cholinergic neurons play a critical role in increasing arousal states during sleep. Recent optogenetic studies have found that activation of cholinergic neurons in the PPT is an important modulator of REM sleep and plays a critical role in the initiation of REM sleep (Van Dort, Zachs et al. 2015).

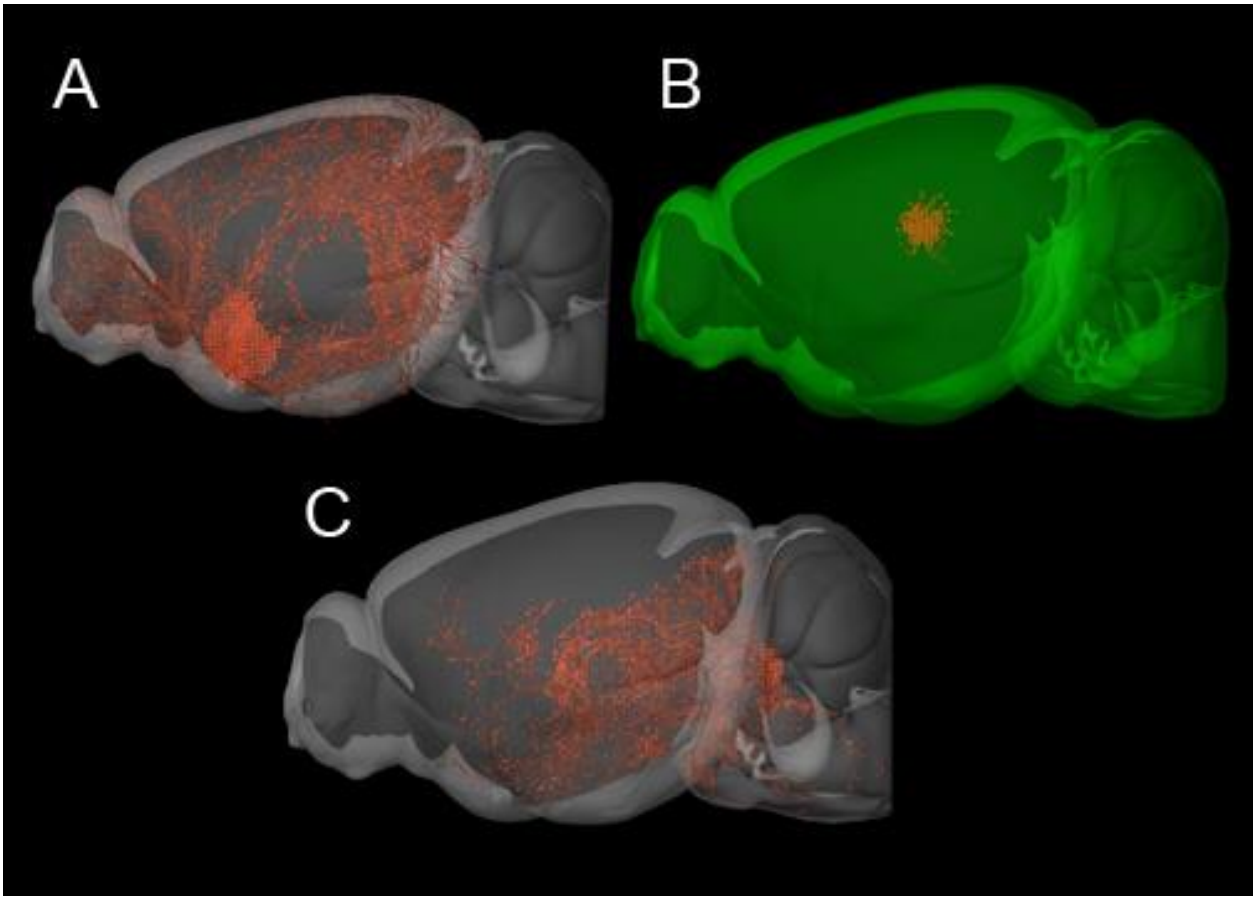


Figure 1.2 The Central Cholinergic System in the Murine Brain. Sagittal 3D reconstructions of the mouse brain. Image data detailing axonal projections labeled by rAAV tracers injections into ChAT-IRES-Cre mice, and visualized using serial two-photon tomography. (A) Cholinergic projections from the basal forebrain. (B) Cholinergic interneurons in the striatum (C) Cholinergic projections from the pedunculopontine tegmental nucleus. Images generated from the Allen Brain Institute Mouse Connectivity Atlas. © 2016 Allen Institute for Brain Science. Allen Mouse Brain Connectivity Atlas [Internet]. Available from: http://connectivity.brain-map.org. Oh, S.W. et al. (2014) A mesoscale connectome of the mouse brain, Nature 508: 207-214. doi:10.1038/nature13186

1.2 Cholinergic Regulation of Brain Functions

1.2.1 Cholinergic Regulation of Hippocampal Function

The hippocampus has been extensively studied for its role in regulating learning and memory (Squire 1992). This brain region plays an important role in the formation and maintenance of new memories. In Alzheimer's disease (AD) the hippocampus is one of the first brain regions to be affected by pathology (De Leon, George et al. 1997, Padurariu, Ciobica et al. 2012). Hippocampal degeneration mediates some of the learning and memory deficits in AD patients (Graham and Hodges 1997).

The finding in humans that drugs which block cholinergic receptors in brain impair performance on task of learning and memory (Ghoneim and Mewaldt 1975, Atri, Sherman et al. 2004), have placed this neurotransmitter as a central regulator of memory. Functional MRI studies have shown that cholinergic modulation of the hippocampus occurs during learning and memory tasks (Goekoop, Scheltens et al. 2006, Wink, Bernard et al. 2006). Much of the work delineating the mechanism of cholinergic control of hippocampal function has been performed in rodents. In both rodents and man, the cholinergic innervation to the hippocampus arises from the medial septum nucleus within the basal forebrain cholinergic system (Lewis and Shute 1967).

The involvement of the basal forebrain cholinergic system in memory is supported by several works employing various methods ranging from unspecific and specific lesion methods (Wrenn, Lappi et al. 1999, Chudasama, Dalley et al. 2004), pharmacological manipulations (Granon, Poucet et al. 1995) and more recently the use of genetic manipulations (Fernandes, Hoyle et al. 2006, Martyn, De Jaeger et al. 2012, Al-Onaizi, Parfitt et al. 2016). The observation of a transient increase of cholinergic activity during and after a memory tasks can be observed in hippocampus (Durkin and Toumane 1992, Durkin 1994). It is suggested that this increase in neurotransmitter release may support synaptic plasticity in the hippocampus, a mechanism which is essential for learning and

memory (Fadda, Melis et al. 1996, Hironaka, Tanaka et al. 2001).

Synaptic plasticity is a process whereby the nervous system changes the weight of functional connectivity; it can help form, eliminate, potentiate and weaken connections of neuronal circuits (Abbott and Nelson 2000). The mechanism of cholinergic regulation of hippocampal plasticity has been well studied (Hasselmo and Bower 1993, McEwen 1999, Yakel and Shao 2004, Hasselmo 2006, Drever, Riedel et al. 2011, Yakel 2012). The addition of low concentrations of carbachol, a cholinergic mimetic, onto cultured slices induces LTP, an effect dependent of muscarinic receptors (Auerbach and Segal 1996). *In vivo* experiments have consistently demonstrated the role of cholinergic tone in the modulation of LTP. Free walking mice showed reduced LTP in the hippocampus, after cholinergic denervation in the medial septum or following administration of muscarinic receptors antagonists (Leung, Shen et al. 2003, Doralp and Leung 2008). Furthermore, deletion of VAChT from basal forebrain neurons also disrupts hippocampal LTP *ex vivo* (Martyn, De Jaeger et al. 2012) and *in vivo* (Al-Onaizi, Parfitt et al. 2016).

The mechanisms by which cholinergic neurons regulate synaptic plasticity are complex and are likely to involve short-term changes in membrane conductance and also on second messengers. Carbachol can induce another type of synaptic plasticity in the hippocampus and the cortex, long-term depression (LTD), once again requiring muscarinic activation (Jo, Son et al. 2010, Caruana, Warburton et al. 2011). LTD is correlated with learning and information storage, furthermore, it has been related to processes which require cognitive flexibility such as extinction and behavior flexibility (Collingridge, Peineau et al. 2010).

Reports have proposed the notion that cholinergic modulation of synaptic plasticity is highly dependent on time, and that nicotinic receptors serve as a switch between LTP and LTD. Ge and Dani (2005), using a protocol to induce a short-term, transient, potentiation (STP) observed that the STP became an LTP response when ACh was administered multiple seconds prior to the stimulation, however when ACh was

delivered less than a second prior the stimulation then the STD turned into a LTD response. Similarly, cholinergic stimulation 120 ms prior to stimulation lead to an α7nAChR dependent LTP. When the same cholinergic stimulation was administered 10 ms after electrical stimulation, a muscarinic dependant LTP was observed (Gu and Yakel 2011).Thus depending on the timing of the activation, either induction of LTP or LTD can occur and will depend on the activation of nicotinic or muscarinic receptors.

Cholinergic activity seems necessary to regulate neuronal synchrony (Metherate, Cox et al. 1992). Neuronal synchrony is the simultaneous change in membrane potential across multiple neurons. This process, in the hippocampus, is thought to underlie many different types of cognitive processing, such as learning and memory (Benchenane, Tiesinga et al. 2011). Furthermore, it has been suggested that disruption of neuronal synchrony may underlie cognitive deficits in diseases such as Alzheimer's and Parkinson's (Uhlhaas and Singer 2006).

1.2.2 Cholinergic Regulation of Attention

Release of ACh from the basal forebrain into the prefrontal cortex (PFC) is essential for attentive processing. ACh signalling in the PFC is an important mechanism for neuromodulation of cortical synaptic activity. The efflux of ACh into the PFC, occurs in rapid transients that act on the millisecond time scale and tonicaly release ACh (Celesia and Jasper 1966). These cholinergic transients are correlated with both cue detection and attentional performance in rodents.

Both selective cholinergic lesions and non-selective lesions of the basal forebrain produce robust deficits in attentional performance in rodents (Robbins, Everitt et al. 1989, Muir, Dunnett et al. 1992, Turchi and Sarter 1997, McGaughy and Sarter 1998). Importantly, the attentional deficits produced by lesions to the basal forebrain cholinergic system are permanent (McGaughy, Kaiser et al. 1996). In addition, these deficits do not alter response times in lesioned animals. On a task where rodents are rewarded for both detecting the presence of salient stimuli and their absence, cholinergic lesions produced drastic deficits in detection of the salient cues, but the rodents had no deficits on trials where they were required to detect the absence of the

stimulus (McGaughy, Kaiser et al. 1996, McGaughy and Sarter 1998). These results implicate basal forebrain cholinergic tone in mediating cue detection. The attention deficits in basal forebrain cholinergic lesioned animals were insensitive to pharmacological administration of nicotinic agonists, suggesting that specifically the release of ACh is critical for cue detection in tasks of attention (McGaughy, Decker et al. 1999).

Microdialysis studies have shown that release of ACh into the PFC is increased when rodents are performing attentional tasks (Passetti, Dalley et al. 2000). Efflux of ACh into the PFC appears to vary based on the difficulty of the attentive task, where the harder the task, the more ACh is released (Dalley, McGaughy et al. 2001). Experiments recording cholinergic signalling in the PFC using enzyme coated microelectrodes demonstrated that attentionally relevant cues correlated with rapid transient increases in ACh levels in the PFC (Parikh, Kozak et al. 2007). These cholinergic transients correlated with attentive behaviour in the animals and were absent when the cues were missed by the animals. Optogenetic stimulation of basal forebrain cholinergic afferents, to simulate cholinergic transients, during tasks of attention produced “false alarms” where rodents behaved as if they had seen a rewarding cue, when no cue had been presented (Gritton, Howe et al. 2016).

Evidence for nicotinic regulation of attention comes from a number of studies which demonstrated the pro-attentive effects of nicotine (Wesnes and Warburton 1984, Rusted and Warburton 1992). The two most prevalent nicotinic receptors in the PFC are the α4β2nAChRs and the α7nAChRs. Evidences from animal models suggest that the effects of nicotine on attention are mediated by α4β2nAChRs (Potter, Corwin et al. 1999, Howe, Ji et al. 2010). Administration of a α4β2nAChRs agonist had a greater effect on attentive behaviour than nicotine (Howe, Ji et al. 2010). Mice lacking the β2nAChR subunit had impairments in attention as assessed by the 5-choice serial reaction time task. These deficits were reversed by viral mediated expression of the β2 subunit specifically in the PFC, demonstrating the necessity and sufficiency of the β2nAChR subunit (Guillem, Bloem et al. 2011). The mechanism of α4β2nAChRs signalling in attention has been thoroughly studied. Stimulation of α4β2nAChRs evoked

release of glutamate into the PFC, lesions of the mediodorsal thalamic nucleus eliminated the glutamate release (Parikh, Man et al. 2008). α4β2nAChRs thus recruit thalamic glutamatergic inputs to regulate cue detection (Howe, Ji et al. 2010). Histological studies have shown that the thalamic glutamatergic nerve terminals in the PFC do indeed express α4β2nAChRs (Lambe, Picciotto et al. 2003).

The evidence for the involvement of α7nAChRs in attention is more contentious. Initial studies suggest that mice null for the α7nAChR have deficits in sustained attention (Hoyle, Genn et al. 2006, Young, Crawford et al. 2007). Others have however reported no deficits in these same mice on the same tasks (Guillem, Bloem et al. 2011). It should be noted that in the latter study the task was substantially easier. The attentional deficits in mice null for the α7nAChR may therefore be dependent on the difficulty of the task assessing attention. Pharmacological manipulations of α7nAChRs have also had mixed results, with some showing improvements in attention and others showing no behavioural effects (Grottick and Higgins 2000, Pichat, Bergis et al. 2007, Rezvani, Kholdebarin et al. 2009, Wallace, Callahan et al. 2011).

The role of muscarinic receptors in attention has been investigated in human studies using the muscarinic inhibitor scopolamine. Administration of scopolamine induces cognitive impairments in healthy human subjects (Dunne and Hartley 1986). The effects of muscarinic blockade on attention appear to involve the visual cortex along with the PFC (Davidson and Marrocco 2000). mAChR blockade in the visual cortex prevents V1 neuronal adaptation to attentional stimuli. Stimulation of mAChRs in the PFC potentiates release of ACh from basal forebrain cholinergic neurons. This top down modulation of attention circuitry by mAChRs plays a role in ignoring distracting stimuli (Sarter, Gehring et al. 2006, Broussard, Karelina et al. 2009). The predominant hypothesis is that attentional performance is the result of synergy between nAChRs and mAChRs. Nicotinic receptors would mediate cue detection and the saliency of the attentionally relevant stimuli, and the muscarinic receptors regulate the top-down processes and recruit the relevant circuitry for sustained attention (Greenwood, Lin et al. 2009).

1.2.3 Cholinergic Regulation of Executive function

Executive function is the umbrella term for the collection of cognitive processes that includes attention, working memory, cognitive flexibility and planning. These cognitive processes allow an organism to monitor their own behaviour and allow for the organism to achieve goal directed behaviour. Impairments in executive function are associated with a number of neurological and neuropsychiatric disorders (Tekin and Cummings 2002).

It is by modulating the activity of various cortical networks, that ACh is believed to regulate executive functioning (Hasselmo and Sarter 2011). Of particular interest, ACh in the PFC is thought to function in a signal-to-noise manner to mediate salient cue detection in the environment (Sarter, Hasselmo et al. 2005). Cortical cholinergic tone has been shown to modulate gamma oscillations (Buhl, Tamas et al. 1998); this effect seems to be dependent of muscarinic activation, mainly by M1 muscarinic receptors (Fisahn, Pike et al. 1998, Fisahn, Yamada et al. 2002). Activation of nicotinic receptors in the PFC has been shown to increase post-synaptic activity (Couey, Meredith et al. 2007). Recently, two-photon imaging has been used to explore the role of nicotinic receptors in the regulation of these effects. Poorthuis and colleagues (2013) have demonstrated that cortical pyramidal neurons can be differentially modulated by α7 and β2 nAChRs depending on the layer of the cortex.

Of importance for executive function and attention, ACh in the PFC appears to regulate both LTP and LTD (Bueno-Junior, Lopes-Aguiar et al. 2012). However, the effects of cholinergic projections arising from the basal forebrain are highly important in synaptic plasticity all throughout the cortex. It regulates both the function and the development of the visual cortex (Hohmann and Berger-Sweeney 1998, Morishita, Miwa et al. 2010). Finally, cortical cholinergic tone is thought to play a role in synchronizing activity in the motor cortex and regulate responses to attentionaly relevant stimuli (Conner, Culberson et al. 2003). The mechanisms underlying the role of cortical cholinergic tone to regulate synaptic plasticity seem to be divergent from those in the hippocampus. For example, in the hippocampus carbachol induction of LTD does not require PLC or PKC activity,

whereas, in the mPFC carbachol-induced LTD requires the participation of PLC and PKC signaling (Huang and Hsu 2010, Caruana, Warburton et al. 2011). Furthermore, cortical cholinergic tone appears to function synergistically with noradrenaline to modulate cortical plasticity (Bear and Singer 1986, Kirkwood, Rozas et al. 1999).

1.2.3.1 Cholinergic Regulation of Cognitive Flexibility

Cognitive flexibility is defined as the ability of higher organisms to adapt under varying environmental conditions, which is essential for survival. In order to test cognitive flexibility in rodents, tasks such as the pairwise visual discrimination are used (Clark, Cools et al. 2004). All tasks which assess cognitive flexibility require the animal to learn a given “rule”, usually a pairing of a stimulus with a reward, then to abandon this rule in favour of a new one. Performance on such behavioural tasks are highly dependent on the PFC. Specifically, the prelimbic subregion of the PFC, has been shown to be important in regulating anxiety-related behaviours, and reversal learning in a visual discrimination task (Heidbreder, Thompson et al. 1996). Direct lesions or temporary inactivation of the prelimbic regions of the prefrontal cortex has no effect on the acquisition of different stimuli, that is to say the initial “rule” but has a robust consequence when a shift in using another strategy is needed in order to a adapt and learn a new “rule” (de Bruin, Sanchez-Santed et al. 1994, Ragozzino, Detrick et al. 1999, Birrell and Brown 2000, Delatour and Gisquet-Verrier 2000, Dias and Aggleton 2000, Ragozzino, Kim et al. 2003). Previous studies have also shown that the striatum, which receives input from the prelimbic region, plays an important role in cognitive flexibility (Dunnett and Iversen 1981, Packard, Hirsh et al. 1989, Knowlton, Mangels et al. 1996, Packard and Teather 1997).

Cholinergic tone in the striatum has been thought to play an important role in mediating cognitive flexibility. Striatal ACh was demonstrated to be critical for regulating synaptic plasticity that underlies forms of learning and memory (Calabresi, Centonze et al. 1998). In addition to the role of ACh in memory functions, Aosaki et al (Aosaki, Tsubokawa et al. 1994) reported that the activity of striatal tonic active neurons was correlated with the presentation of rewards, providing insight on the role of striatal ACh in goal-oriented

behaviors. However, the exact role of striatal ACh in cognitive flexibility is still under investigation. Mice with striatal interneurons deficient for VAChT showed no deficits in a water maze based reversal learning paradigm (Guzman, De Jaeger et al. 2011), a form of cognitive flexibility.

Results from mice lacking forebrain VAChT indicate that cholinergic tone is not required for the initial spatial learning phase in a Morris Water Maze task. However, when the contingencies are changed, and the platform is moved to a different quadrant of the pool, these mutant mice are completely unable to adapt and learn the new “rule”, suggesting that forebrain cholinergic tone is required for such behaviour (Martyn et al., 2012). Although there is a clear role of ACh in cognitive flexibility little is known of the mechanisms by which ACh exerts its effect.

Administration of the M1 muscarinic antagonist scopolamine is one of the most robust means by which impairments in behavioural flexibility can be induced (Ragozzino, Jih et al. 2002, Klinkenberg and Blokland 2010). Furthermore administration of agonists and positive allosteric modulators of M1 improve performance on measures of cognitive flexibility (McCool, Patel et al. 2008, Shirey, Brady et al. 2009). Interestingly, visual discrimination and reversal learning tested in mice deficient for the M1 muscarinic receptor showed no significant differences from control mice on acquisition or reversal (Bartko, Romberg et al. 2011), in contrast to the pharmacological data. Aging induced impairments in cognitive flexibility has been correlated with decreased expression of the M2 muscarinic receptor. Seeger *et al* (2004), investigated the role of M2 receptors, using M2 knockout mice in behavioural tasks assessing working memory and behavioural flexibility. They reported that M2 null mice had deficit in the acquisition phase of the Barnes Maze by their inability to switch from a random strategy to a more efficient strategy. Thereby, concluding that cognitive flexibility could be mediated through M2 receptors.

The role of nicotinic receptors in cognitive flexibility has not been greatly explored. Nicotinic ACh receptors, specifically the α7nAChR, have been shown to alter both release of glutamate in the PFC (Marchi, Risso et al. 2002, Gomez-Varela and Berg 2013) and trafficking of glutamate receptors (Yang, Paspalas et al. 2013). As Glutamate

signalling (Karlsson, Tanaka et al. 2009) and glutamate receptor trafficking (Brigman, Feyder et al. 2008, Brigman, Daut et al. 2013) play important roles in cognitive flexibility, it is possible that cholinergic modulation of glutamate signalling, via nicotinic receptor activation, may contribute to one's ability to reverse contingency rules and demonstrate cognitive flexibility.

1.3 Alzheimer's Disease

AD is a chronic neurodegenerative disease which is characterized by loss of neurons and synapses in cortical and subcortical brain regions. This cell loss results in atrophy of the effected brain regions. This neuropathology manifests itself symptomatically early on as memory loss, but as the disease progresses the symptoms expand to include long term memory loss, impairments in executive functioning and mood disorders. The pathological hallmarks of the disease are extracellular amyloid plaques, and intracellular neurofibrillary tangles. This pathology has been hypothesised to play a critical role in disease development, however the etiology of the disease remains poorly understood.

1.3.1 Amyloid Pathology in AD

A pathological hallmark of AD, is the appearance of extracellular amyloid plaques. The presence of these plaques are a required diagnostic criteria for AD and plaque burden within the brain is used to determine the Braak staging of AD (Braak, Braak et al. 1986). These amyloid plaques are composed of aggregated fragments of the amyloid precursor protein (APP) (Weidemann, Konig et al. 1989).

APP is an integral membrane protein and within the brain its expression is highest at synapses (Muller and Zheng 2012, Puig and Combs 2013). Mice deficient in the APP gene present subtle cognitive deficits and electrophysiological changes but do not recapitulate an "Alzheimer's-like" phenotype (Zheng, Jiang et al. 1996, Mileusnic, Lancashire et al. 2000, Wang, Yang et al. 2005). It is therefore understood that the amyloid toxicity in the AD brain arises from a gain of function of the protein rather than a loss of the protein normal functions. The normal cellular function of the amyloid precursor protein remains unclear. The protein has been linked to many key neuronal

functions including synaptic formation (Priller, Bauer et al. 2006), neuronal transport (Torroja, Chu et al. 1999), iron export (Rogers, Bush et al. 2008) and hormonal signalling (Bandyopadhyay, Goldstein et al. 2007).

APP under normal conditions undergoes a series of post-translational modifications, most notably proteolytic cleavage of the protein. APP can undergo either an amyloidogenic or a non-amyloidogeneic proteolytic cleavage, these cleavage pathways are mediated by distinct proteolytic enzymes. The non-amyloidogeneic pathway begins with APP cleavage by α-secretase into a soluble APPα fragment and a C83 fragment (Esch, Keim et al. 1990, Sisodia, Koo et al. 1990). The C83 fragment is subsequently cleaved by the γ-secretase enzyme to form the non-toxic P3 (Haass, Hung et al. 1993) and the APP protein's intracellular domain or AICD C-terminus fragments (Gu, Misonou et al. 2001).

Amyloidogeneic proteolytic cleavage of APP is initiated by the β-secretase (BACE1) enzyme (Cai, Wang et al. 2001). Given its role in initiation of toxic APP processing, BACE1 is the rate limiting step in Aβ production (Stockley and O'Neill 2007). BACE1 will cleave APP into soluble APPβ fragments and C99 fragments (Luo, Bolon et al. 2001). It is these C99 fragments which can be cleaved at a number of sites by the γ-secretase enzymatic complex to produce Aβ species and AICD fragments (Citron, Westaway et al. 1997, Dovey, John et al. 2001). The cleavage pathway by which APP is catabolised is determined by the relative abundance of the α- and β-secretases. However mutations in both the APP gene (De Jonghe, Esselens et al. 2001) and in the secretase enzymes can favour production of Aβ (Yan, Bienkowski et al. 1999, Jankowsky, Fadale et al. 2004). Changes in the catabolism of APP to produce Aβ-proteins is thought to trigger the amyloid cascade in the AD brain and is the basis of the amyloid hypothesis of AD (Hardy and Higgins 1992). There are three predominant forms of Aβ peptides: Aβ38, Aβ40 and Aβ42 (Portelius, Tran et al. 2007). Additionally, an Aβ43 peptide has been described in presenilin-1 mutation knockin mice (Xia, Kelleher et al. 2016). There is evidence that the more hydrophobic forms of these peptides, in particular the Aβ42, are

more related to severity of AD (Chui, Tanahashi et al. 1999). Specifically, the ratio of Aβ42 to Aβ40 is most related to disease severity (Jarrett, Berger et al. 1993).

A key component therefore in Aβ production is the γ-secretase enzymatic complex. This enzymatic complex is composed of four essential core proteins (Edbauer, Winkler et al. 2003): presenilin-1(Haass and Steiner 2002), nicastrin (Yu, Nishimura et al. 2000), APH-1 (Goutte, Tsunozaki et al. 2002) and presenilin enchancer-2 (Francis, McGrath et al. 2002). Mutations in these core proteins enhance production of Aβ42 peptides (Citron, Westaway et al. 1997, Yu, Nishimura et al. 2000). Inhibition of γ-secretase on the other hand reduces beta-amyloid production in the brain (Dovey, John et al. 2001). The individual core proteins have also been shown to play regulatory roles in APP processing beyond their enzymatic activity. Presenilin-1 for example can modulate APP processing by regulating the intracellular trafficking of the protein (Leem, Saura et al. 2002, Cai, Leem et al. 2003). Depictions of the amyloidogenic and non-amyloidogeneic APP cleavage pathways are shown in Figure 1.3.

Given the highly neurotoxic nature of Aβ-oligomers and potentially of Aβ plaques, many therapeutic approaches have been developed to target the production of Aβ, by inhibiting BACE1(Vassar 2001) or the γ-secretase complex (Dovey, John et al. 2001). Although targeting these enzymatic pathways may be promising therapeutic avenues, concerns have been raised that interfering with these enzymes may be detrimental to neuronal health (Hu, Hicks et al. 2006, Haapasalo and Kovacs 2011). The γ-secretase enzymatic complex for instance, plays many other critical roles in the cell including Notch processing (De Strooper, Annaert et al. 1999). Therefore, the general inhibition of this complex has been shown not to be a good approach for therapeutic interventions for AD (Haass and Selkoe 2007). Targeting the amyloidogenic activity of the complex while leaving the Notch signalling unaffected can be achieved by modulating proteins which activate the complex (Netzer, Dou et al. 2003, He, Luo et al. 2010).

Total Aβ levels, particularly the Aβ42 molecule, in the brain can either be elevated by increase in production of the toxic species or by a reduction in their clearance (Selkoe 1993, De Felice and Ferreira 2002). This increase in Aβ-proteins leads to the

propensity to form Aβ-oligomers in the brain (Lambert, Barlow et al. 1998, Walsh, Klyubin et al. 2002, Gong, Chang et al. 2003). The oligomers disrupt synaptic function (Wang, Pasternak et al. 2002, Lacor, Buniel et al. 2004, Lacor, Buniel et al. 2007). Eventually the accumulation of these oligomers triggers amyloid plaque formation (Ahmed, Davis et al. 2010) and elicits an inflammatory response (White, Manelli et al. 2005, Sondag, Dhawan et al. 2009). This accumulation of Aβ-oligomers therefore leads to progressive neuronal injury (Zhang, McLaughlin et al. 2002). Aβ-oligomers have also been shown to trigger tau hyperphosphorylation (Ma, Yang et al. 2009). These events together have been suggested to underlie neuronal dysfunction and cognitive decline in the AD brain (Cleary, Walsh et al. 2005, Tomic, Pensalfini et al. 2009).

Aβ-oligomers can produce cytotoxicity by a number of mechanisms. A number of transmembrane receptors have been identified that Aβ-oligomers can bind to, including the NMDA receptor (Texido, Martin-Satue et al. 2011), α7nAChR (Wang, Lee et al. 2000, Lilja, Porras et al. 2011), insulin(Zhao, De Felice et al. 2008) and the PrP^c (Lauren, Gimbel et al. 2009, Chen, Yadav et al. 2010). Binding to these membrane proteins can trigger a number of cytotoxic signalling pathways, ultimately leading to cell death (Haass and Selkoe 2007). Aβ-oligomers have also been proposed to increase membrane permeability and by doing so, disrupt the cationic homeostasis of the affected cells (Lin, Bhatia et al. 2001, Demuro, Mina et al. 2005). Aβ-oligomers can also trigger cytotoxic cellular signalling when it accumulates within neurons. These intracellular aggregates of Aβ have been shown to impair proteasome function (Tseng, Green et al. 2008), disrupt mitochondria (Manczak, Anekonda et al. 2006) and increase production of toxic reactive oxygen species (Cenini, Cecchi et al. 2010).

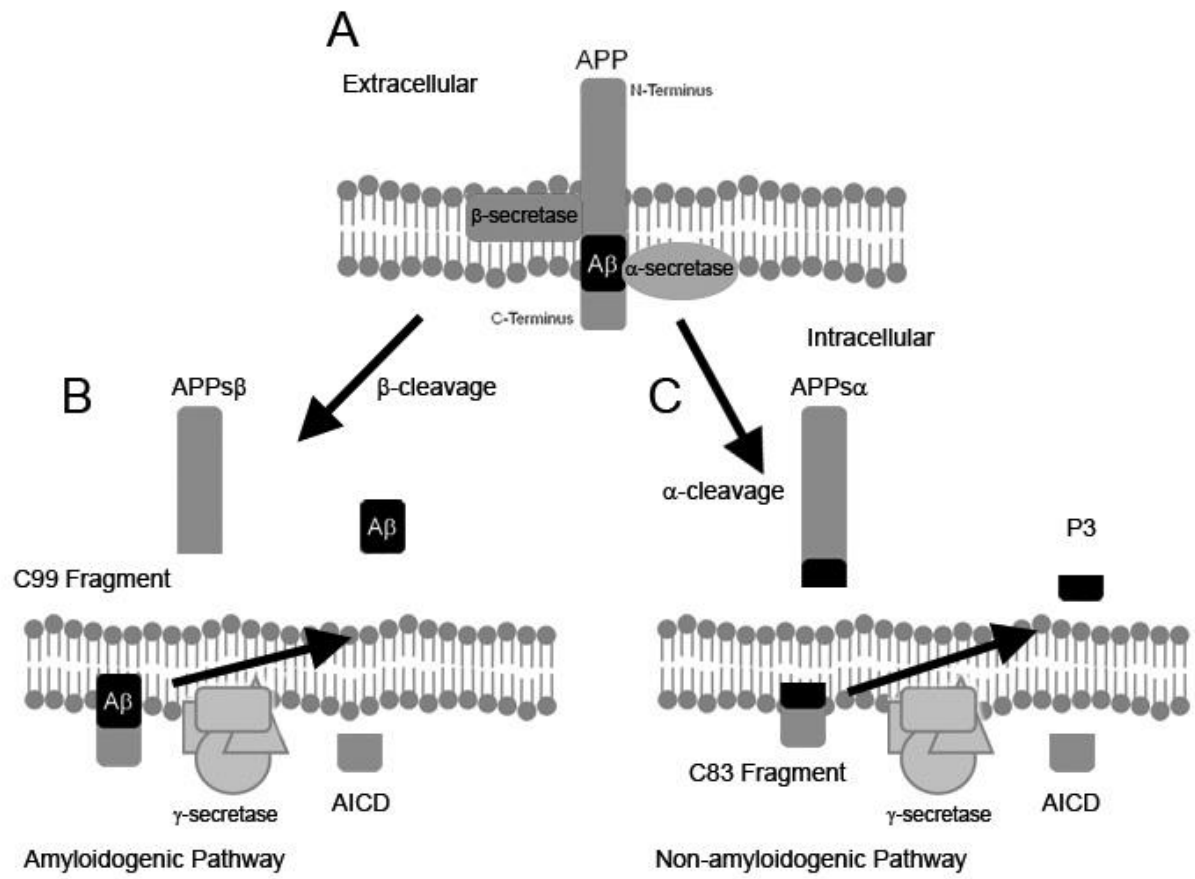


Figure 1.3 Processing of the Amyloid Precursor Protein. (A) Transmembrane APP protein can be cleaved by either the α-secretase or the β-secretase enzymes. Cleavage by the β-secretase enzyme leads to the amyloidogenic processing of the protein, while cleavage by the α-secretase leads to the non- amyloidogenic processing of the protein. (B) The amyloidogenic processing of APP. Following cleavage by the β-secretase enzyme a β soluble APP fragment and a C99 fragment of the protein are formed. The C99 fragment undergoes subsequent cleavage to form toxic Aβ fragments and AICD fragments. This cleavage is mediated by the γ-secretase complex. (C) The non-amyloidogenic processing of APP. Following cleavage by the α-secretase enzyme an α soluble APP fragment and a C83 fragment of the protein are formed. The C83 fragment undergoes subsequent cleavage to form inert P3 fragments and the AICD fragment. This cleavage is mediated by the γ-secretase complex.

1.3.2 Tau Pathology in Alzheimer's Disease

Another key pathological hallmark of AD, is the appearance of intracellular neurofibrillary tangles (NFTs). Inclusion of NFTs, along with amyloid plaques, are a diagnostic criteria for AD and the severity of the NFT pathology are also important for the Braak staging of AD (Braak, Braak et al. 1986). NFTs are composed of aberrantly phosphorylated microtubule associated protein tau (Goedert, Wischik et al. 1988). The exact relationship between tau pathology and neurodegeneration remains unclear. Specifically, it is not fully understood if tau pathology arises from the loss of the protein normal function, or if tau hyperphosphorylation leads to a toxic gain of function of the protein.

The full length tau protein is 441 amino acid residues long and presents two characteristic domains. Near the C-terminus of the protein, there are a number of tubulin binding domains, and near the N-terminus there are two acidic domains. The predominant function of the tau protein is to stabilize microtubules (Lee, Cowan et al. 1988). This function is mediated by the two characteristic structural domains of the protein, with the tubulin binding domain binding β-tubulin (Maccioni, Rivas et al. 1988), and the acidic domains binding to the negatively charged regions of microtubules (Ennulat, Liem et al. 1989). This interaction between tau and microtubules can be regulated by phosphorylation of the tau protein. Phosphorylation of tau negatively regulates its interaction with microtubules (Steiner, Mandelkow et al. 1990). Aberrant phosphorylation of the tau protein can thus lead to microtubule instability, a potential mechanism of tau neurotoxicity.

The effects of tau phosphorylation are dependent on which amino acid residues on the protein are phosphorylated. Different phosphorylation sites can have different physiological or pathological outcomes. Tau phosphorylation plays an important role in regulating microtubule dynamics in neurons, however when deregulated this process can become neurotoxic. The key inhibitory phosphorylation sites on tau are Ser262, Thr231, and Ser235; tau phosphorylated at any of these amino acids will have inhibited

microtubule binding capacities (Sengupta, Kabat et al. 1998). Furthermore, phosphorylation at Ser199/Ser202/Thr205, Thr212, Thr231/Ser235, Ser262/Ser356, and Ser422, will not only inhibit the function of the protein, but will cause tau to sequester normal tau proteins away from microtubules and inhibit their function as well (Alonso Adel, Mederlyova et al. 2004). Finally phosphorylation at Thr231, Ser396, and Ser422 have also been shown to promote self-aggregation of the tau in vitro (Abraha, Ghoshal et al. 2000).

Phosphorylation of tau is a dynamic process with a number of kinases and phosphatases interacting with the protein. The kinases which have been identified to phosphorylate tau are glycogen-synthase kinase-3β (GSK-3β) (Takashima, Noguchi et al. 1993), cyclin-dependent protein kinase 5 (Baumann, Mandelkow et al. 1993), cAMP-dependent protein kinase (Jicha, Weaver et al. 1999),and stress-activated protein kinases (Ferrer, Gomez-Isla et al. 2005). These kinases preferentially phosphorylate tau at different amino acid residues. GSK-3β in particular has been identified as promoting toxic phosphorylation of tau. This kinase will preferentially phosphorylate at Ser202/Thr205, Ser214/Thr212, Thr231 and Ser396 residues (Song and Yang 1995). PP2A has been identified as the major phosphatase of tau (Goedert, Jakes et al. 1995). Importantly, activity and expression of this phosphatase has been shown to be decreased in the AD brain (Liu, Grundke-Iqbal et al. 2005).

Tau neurotoxicity is likely the result of both the gain and loss of functions resulting from hyperphosphorylated tau. As described above, when tau is hyperphosphorylated it no longer stabilizes microtubules. Hyperphosphorylation of the protein can therefore destabilize microtubules and compromise axonal transport (Zhang, Maiti et al. 2005). Furthermore, tau hyperphosphorylation causes the protein to bind to its un-phosphorylated form and inhibits its own function. This again leads to microtubule instability and compromised axonal transport (Zhang, Maiti et al. 2005). Microtubule instability is highly toxic to neurons (Gornstein and Schwarz 2014), drugs that inhibit microtubule stability are toxic to cultured neurons within hours and can cause neurological impairments within a week *in vivo* (Pisano, Pratesi et al. 2003).

1.3.3 Cholinergic Vulnerability in Alzheimer's Disease

A central hypothesis in AD, is that the cognitive symptoms of the disease arise as a result of the loss of basal forebrain cholinergic signalling. It was first reported in the 1970's that anticholinergic drugs could impair cognitive function in humans (Deutsch 1971). Given these findings, it was hypothesized that this neurotransmitter system may be dysfunctional in dementia. Post mortem analysis of AD brains has revealed significant reductions in levels of the ChAT enzyme in the cortex (Bowen, Smith et al. 1976, Perry, Gibson et al. 1977). Furthermore, it has been shown that in the AD brain there is a reduction in choline uptake (Rylett, Ball et al. 1983), ACh release (Nilsson, Nordberg et al. 1986), VAChT (Efange, Garland et al. 1997, Chen, Reese et al. 2011, Parent, Bedard et al. 2013) and an overall reduction in the number of cholinergic neurons in the basal forebrain region (Davies and Maloney 1976, Whitehouse, Price et al. 1982). This body of evidence lead to the cholinergic hypothesis of AD (Bartus, Dean et al. 1982). This hypothesis is the basis for the use of cholinesterase inhibitors as standard therapy for AD. These drugs have been shown to be effective in improving behavioural symptoms of the disease and delaying the placement of the patients in nursing homes (Cummings 2003).

One of the hypothesis of AD is that the neuronal dysfunction that occurs in the disease precede the cognitive decline (Nestor, Scheltens et al. 2004). That is, the pathological changes observed in the brain cause neuronal dysfunction and cell loss, leading to cognitive decline. It is therefore important to detect AD prior to the clinical manifestation of the disease. It was reported that there was no change in ChAT or AChE levels in the cortex of individuals with mild-AD (Davis, Mohs et al. 1999). Furthermore, the activity of the enzyme was reported as unaltered in the mild-AD brain and activity was increased in the brain of patients with mild cognitive impairment (DeKosky, Ikonomovic et al. 2002). These findings led to criticisms of the validity of the cholinergic hypothesis of AD (Morris 2002). A strong counterargument to these criticism, however is that neither total levels nor activity of the ChAT or AChE enzymes represent accurate measures of ACh release. Therefore the levels of these enzymes in the AD brain are not necessarily

indicative of changes in cholinergic signalling (Terry and Buccafusco 2003). Further evidence of cholinergic dysfunction (either for or against), in particular assessment of ACh release, in early AD is therefore still needed.

The main risk factor for AD is aging (Lindsay, Laurin et al. 2002). Therefore, numerous studies have examined the effect of aging on the cholinergic system. It was shown that in mice, ACh synthesis is reduced by 75% when comparing young (3 months old) to aged (30 months old) animals (Gibson, Peterson et al. 1981). Furthermore, as mice age, their cholinergic neurons no longer release as much ACh as their younger counterparts (Gibson and Peterson 1981). These findings were confirmed in rats as well (Wu, Bertorelli et al. 1988). Numerous studies in animals have suggested that cholinergic neurons function “normally” and that the aging process stresses these neurons and decreases their function (Meyer, Crews et al. 1986, Gilad, Rabey et al. 1987, Moore, Stuckman et al. 1996). Accelerating the aging-induced deficits in cholinergic signalling has been shown to have functional consequences in rat model of sustained attention (Burk, Herzog et al. 2002).

Recent evidence has added a new dimension to the cholinergic hypothesis of AD. Clinical findings report that there is an increased incidence of dementia in patients following the cumulative use of anticholinergic drugs (Gray, Anderson et al. 2015, Risacher, McDonald et al. 2016). It is well understood that the use of these compounds can impair cognition while the patient is taking them. However, there is a growing body of clinical evidence suggesting that anticholinergic medications can produce cognitive impairments long after consumption of the medications has been cessed (Carriere, Fourrier-Reglat et al. 2009, Jessen, Kaduszkiewicz et al. 2010). Furthermore, it has been reported that prolonged use of these drugs, specifically anti-muscarinic agents, increases Alzheimer’s pathology in the brains of these patients (Perry, Kilford et al. 2003). *In vitro* models, have confirmed that anti-muscarinic agents have the most potent effect on neuronal and astrocytic cells (Woehrling, Parri et al. 2015). Mechanistic insight, however, into how anticholinergic medications can increase risk of dementia are still largely unknown.

Cholinergic neurons also appear to be highly vulnerable to AD pathology. Post-mortem analysis of AD brains has shown that basal forebrain cholinergic neurons present with both amyloid (Baker-Nigh, Vahedi et al. 2015) and tau pathology (Vana, Kanaan et al. 2011, Ahmadian, Rezvanian et al. 2015). These cholinergic basal forebrain neurons have been shown to be vulnerable to both of these AD related pathologies. This selective vulnerability is mediated by the dependence of these basal forebrain cholinergic neurons on trophic factors. Aβ species impair trophic signalling in these neurons, resulting in impairment in their function and toxicity to the cells (Cuello and Bruno 2007). Targeting these neurotrophic pathways in mouse models of AD can improve cognitive outcomes in these mice, and at the same time reduce tau and Aβ related pathology (Nguyen, Shen et al. 2014). Furthermore, it has been shown that Aβ species promote tau hyperphosphorylation in basal forebrain cholinergic neurons (Zheng, Bastianetto et al. 2002). Cholinergic neurons are therefore highly vulnerable to AD pathology and this is thought to play a role in mediating cognitive dysfunction in the disease. Also, the magnitude of the loss of these neurons correlates to cognitive deficits in patients (Mufson, Ma et al. 2002). It should be noted that other factors have been shown to correlate to cognitive dysfunction including Aβ oligomers (Cleary, Walsh et al. 2005, Tomic, Pensalfini et al. 2009) and general synaptic dysfunction in the AD brain (DeKosky and Scheff 1990, Selkoe 2002).

Aberrant cholinergic signalling can also impact the signalling pathways related to both tau and amyloid pathology. Immunolesions of basal forebrain cholinergic neurons in the 3xTG mouse model of AD (which has both amyloid and tau pathology), significantly enhanced pathology in these mice (Hartig, Saul et al. 2014). Furthermore administration of an M1 muscarinic agonist to 3xTG mice significantly decreased the pathology and hippocampal specific memory impairments in the animals (Caccamo, Oddo et al. 2006). These effects were purported to be mediated by regulating GSK3 activity and BACE1 expression. Crossing Alzheimer's model mice with mice lacking the M1 muscarinic receptor significantly increased amyloidogenic processing of APP and accumulation of toxic Aβ species (Davis, Fritz et al. 2010). Further work in mice has shown that

impairing cholinergic signalling accelerates tau hyperphohsphorylation (Zhang, Chen et al. 2014). These studies speak to the complexity of AD pathology and provide evidence that cholinergic signalling, along with being highly vulnerable to AD pathology, may also be important in regulating AD pathology.

1.3.4 RNA Metabolism Dysfunction in Alzheimer's Disease

RNA metabolism refers to any events in the life cycle of an RNA molecule, including its synthesis, modification, processing, translation and ultimately its degradation (Stamm, Ben-Ari et al. 2005). Alternative splicing is the process by which the mRNA of a given gene can be edited to form differential transcripts. This process involves the inclusion or the exclusion of particular exons or introns in a transcript (Zahler, Neugebauer et al. 1993). Alternative splicing is a central aspect of neural physiology and is carried by RNA binding proteins. The function of a number of RNA binding proteins is therefore essential for both the development and function of neurons (Jensen, Dredge et al. 2000).

Alternative splicing of mRNAs prior to their translation is an essential regulatory process in eukaryotic cells. It is by alternative splicing that a single gene composed of multiple exons can be translated into proteins which have different structures and functions (Padgett, Grabowski et al. 1986, Breitbart, Andreadis et al. 1987). Furthermore, the alternative splicing of an mRNA plays a critical role in the final cellular localization of the gene product. For example, if a nuclear localization signal of a given protein is added by alternative splicing that protein will now translocate to the nucleus, as is the case with CaM-Kinase (Srinivasan, Edman et al. 1994). Alternative splicing plays a critical role in both the development and in the normal function of the mammalian nervous system (Li, Lee et al. 2007, Raj and Blencowe 2015).

There are multiple patterns of alternative splicing that have been identified in eukaryotic cells (Sammeth, Foissac et al. 2008). Schematic depictions of the different forms of alternative splicing can be found in Figure 1.4. The most common alternative splicing event is the cassette-exon event (Sammeth, Foissac et al. 2008); this is the inclusion or exclusion of a single exonic sequence from the final mRNA transcript (Van der Ploeg,

Liu et al. 1982). A mutually exclusive exon splicing event is when the inclusion of a given exon in a transcript results in the exclusion of another (usually a proximal exon) (Smith and Nadal-Ginard 1989). Alternative splicing can also occur within an exon. Splicing of a sequence which changes the length of the exon at the 5'- end is an alternative 5' splicing event, and an event that does the same at the 3' end of an exon is a 3' alternative splicing event (Fu, Mayeda et al. 1992). Intronic sequences are usually spliced out of a final transcript, however alternative splicing of an mRNA can result in the retention of intronic sequences (Galante, Sakabe et al. 2004). Alternative splicing can also occur within the promoter region of a given transcript, when this event occurs it results in a different first exon for the final transcript (Cramer, Caceres et al. 1999). Alternative splicing also plays a significant role in the regulation of the 3' UTR of a transcript, as it can result in a completely different 3'-UTR (Grimm, Holinski-Feder et al. 1998). The length of the 3'UTR is also prone to alternative splicing, these events are termed alternative polyadenylation events (Proudfoot, Furger et al. 2002). Alternative polyadenylation of a transcript is an important determinant of which microRNA end up binding to the transcript and therefore can effect translation and degradation of the transcript (Sandberg, Neilson et al. 2008).

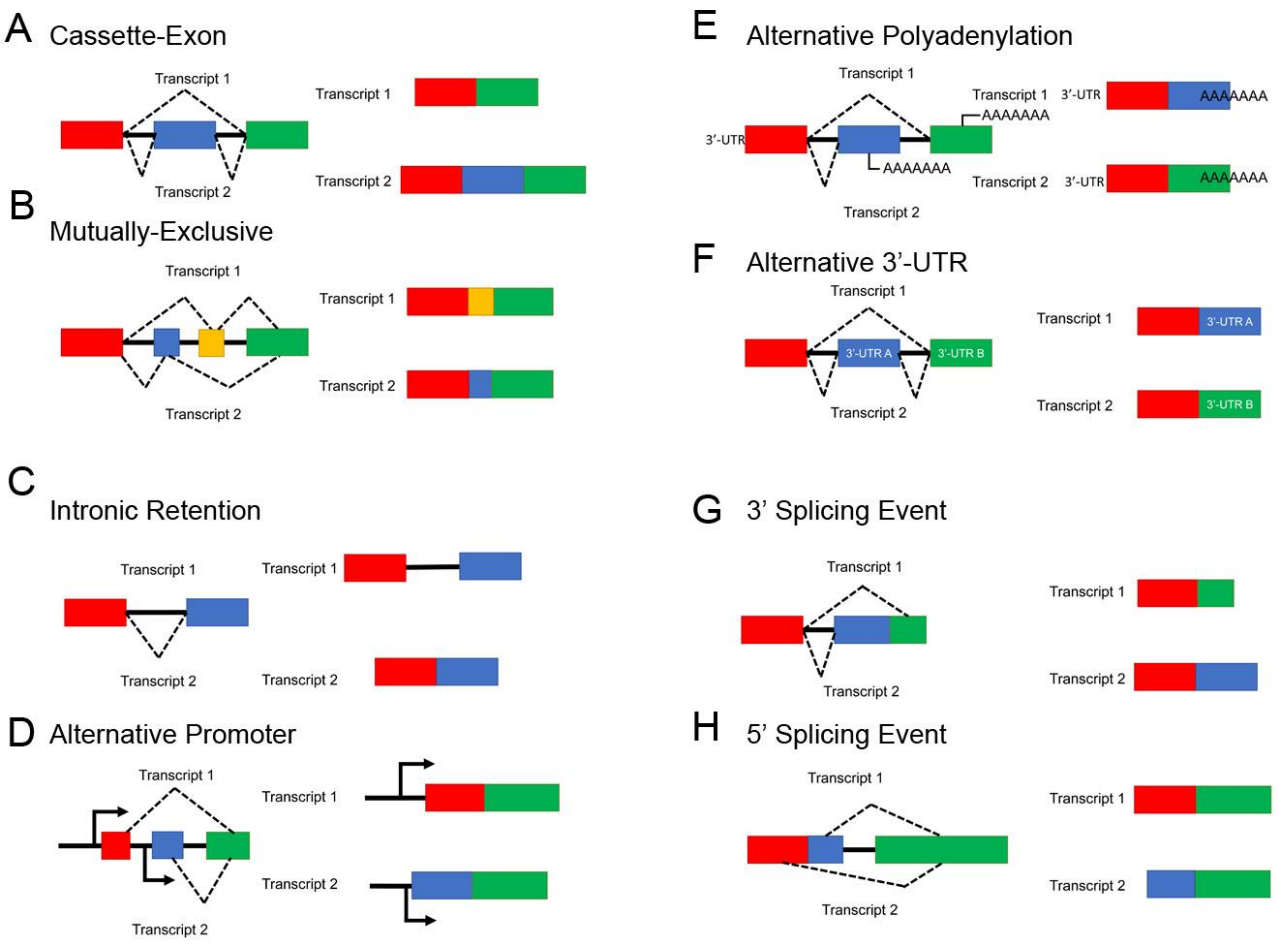


Figure 1.4. Patterns of alternative splicing in eukaryotic cells. (A) Cassette-exon splicing events are the inclusion or exclusion of a single exonic sequence from the final mRNA transcript. (B) Mutually-exclusive splicing events occur when the inclusion of a given exon in a transcript results in the exclusion of another. (C) Intronic retention, when an intronic sequence is retained in the final mRNA transcript. (D) Alternative promoter events are when alternative splicing changes the first exon of a given transcript. (E) Alternative polyadenylation events occur when the length of the 3'-UTR of a transcript is altered. (F) Alternative 3'-UTRs occur when the 3'UTR of a transcript is completely alerted by alternative splicing. (G) 3'-Splicing event. When alternative splicing occurs within the 3' region of an exon. (H) 5'-Splicing event. When an alternative splicing event occurs within the 5' region of an exon.

All of these different patterns of alternative splicing occur concurrently on transcripts within eukaryotic cells. These splicing events are regulated by hosts of RNA-binding

proteins (RBPs) (Mount, Pettersson et al. 1983, Mayeda and Krainer 1992, Wu and Maniatis 1993, Izaurralde, Lewis et al. 1994). RBPs all bind to RNA and either enhance or repel spliceosome formation and ultimately the alternative splicing of specific sequences (Blencowe 2000). Any given RBP can have different effects on a single transcript based on where the RBP binds to the mRNA, post-translational modification to the RBP and interactions with other RBPs (Tacke, Chen et al. 1997, Xiao and Manley 1997, Black 2003). In the mammalian nervous system, there is a significant amount of overlap between the functions of the various RBPs. This creates a complex and finely tuned regulatory network regulating RNA metabolism within each neuron in the brain (Vuong, Black et al. 2016). The interactions between these RNA binding proteins in neurons is complex and plays a vital role in neuronal function (McGlincy and Smith 2008) and are dysfunctional in AD (Bai, Hales et al. 2013).

Many familial mutations have been identified in key AD related genes that cause alternative splicing of these transcripts. This includes the genes for the amyloid precursor protein (Golde, Estus et al. 1990) and tau (Niblock and Gallo 2012). Furthermore mutations in genes involved in RNA metabolism are central to many familial forms of neurodegeneration, including AD (Lemmens, Moore et al. 2010). In particular, dysfunction of RNA metabolism is an important factor in the etiology of Amyotrophic Lateral Sclerosis (ALS). Many of the key processes in RNA metabolism have been shown to be disrupted in ALS (Strong 2010). Microarray analysis has shown that a large number of key transcripts are both up and down regulated in ALS, suggesting impaired regulation of transcription in the disease (Ferraiuolo, Heath et al. 2007). Furthermore, there is evidence of altered alternative splicing(Rabin, Kim et al. 2010), mRNA transport deficits (Millecamps and Julien 2013) and impairment in RNA translation in ALS (Ling, Polymenidou et al. 2013). Mutations in hnRNPA2/B1 and hnRNPA1 (see below) have been associated with familial forms of ALS. Importantly, these mutations result in the proteins aggregating in stress granules and forming cytoplasmic inclusions (Kim, Kim et al. 2013).

The role of alternative splicing in sporadic AD however is not well defined. The hippocampus was identified as a region vulnerable to changes in RNA metabolism in

the sporadic AD brain. Importantly, these observed changes in RNA metabolism, were uncorrelated to pathological hallmarks of AD (Doebler, Markesbery et al. 1987). Many studies have demonstrated that transcriptome level changes in genes related to neuronal function are a hallmark of AD (Miller, Oldham et al. 2008). Microarray studies of the AD brain temporal lobe found that there was significant amounts of alternative splicing, and that this was reflected by changes in RNA binding protein levels (Tollervey, Wang et al. 2011). Further microarray analysis of post-mortem entorhinal cortices from AD and non-demented control brains, found global changes in alternative splicing in the sporadic AD brain (Berson, Barbash et al. 2012). Importantly, these changes in alternative splicing were linked to hnRNPA/B proteins.

1.3.4.1 hnRNPA/B Proteins

Heterogeneous nuclear ribonucleoproteins (hnRNP) are a large family of protein which associate with and bind pre-mRNA into hnRNP particles. hnRNP particles are complexes of mRNA and protein. A mRNA being in an hnRNP particle indicates to the cell that the mRNA is not mature, and is not ready for translation (Konig, Zarnack et al. 2010). It is within this hnRNP particle that splicing of the mRNA occurs. A subfamily of the hnRNP proteins is the A/B family. The hnRNPA/B family is comprised primarily of the hnRNPA1 hnRNPA2/B1 proteins (Minoo, Martin et al. 1991), although hnRNPA0 (Myer and Steitz 1995) and hnRNPA3 (Matsui, Motomura et al. 2002) are also members of the family. All hnRNPA/B proteins have two RNA-recognition motifs within a glycine rich domain (Dreyfuss, Matunis et al. 1993). All proteins within the family have a relatively high level of homology between these RNA-recognition motifs (Burd, Swanson et al. 1989). hnRNPA/B proteins are the most abundant hnRNP proteins within cells. hnRNPA2/B1 and A1 comprise roughly 60% of all hnRNP proteins within cells (Beyer, Christensen et al. 1977). hnRNPA/B proteins are most abundant within the nucleus of cells (Pinol-Roma, Swanson et al. 1989), however the shuttling of these proteins between the nucleus and the cytoplasm is critical for many of their functions.

The key function of hnRNPA/B proteins is their involvement is RNA-editing. hnRNPA/B proteins are essential components of the spliceosome and are involved in alternative and constitutive splicing events (Jurica, Licklider et al. 2002). hnRNPA/B proteins do not directly splice mRNA, rather, they regulate alternative splicing by attracting or repulsing serine/arginine-rich (SR) proteins (Martinez-Contreras, Cloutier et al. 2007). Therefore, hnRNPA/B proteins can act as either splicing enhancers or repressors, depending on their RNA-recognition motif and the element they bind on a given mRNA, and their effect on SR proteins.

hnRNPA/B proteins can also play important roles in other aspects of RNA metabolism. These proteins can play a role in gene transcription, by binding to promoter regions of genomic DNA (Takimoto, Tomonaga et al. 1993, Campillos, Lamas et al. 2003). The binding of these hnRNPA/B proteins can either promote or inhibit transcription of genes. hnRNPA/B proteins also play a role in shuttling mRNA out of the nucleus. hnRNPA/Bs interact with transportin 1 and 2 proteins (Rebane, Aab et al. 2004) and is then be exported from the nucleus by nucleoporins (Bonifaci, Moroianu et al. 1997). hnRNPA/Bs then shuttle mRNAs to polysomes for translation (Visa, Alzhanova-Ericsson et al. 1996). In neurons this can involve transporting key transcripts, in an activity dependant manner, from the nucleus to the synapse, such as *BDNF* mRNA (Leal, Afonso et al. 2014). hnRNPA2/B1 is also found to bind to, and interact with miRNAs. hnRNPA2/B1 will specifically bind miRNAs which have a recognition element for hnRNPA2/B1, the protein controls the loading of miRNAs into exosomes (Villarroya-Beltri, Gutierrez-Vazquez et al. 2013).

1.3.4.2 hnRNPA/B Proteins in AD

The selective loss of hnRNPA/B proteins in the AD brain was an important breakthrough in understanding the mechanism which regulate alternative splicing (Berson, Barbash et al. 2012). In mice, viral mediated knockdown of hnRNPA/B proteins recapitulated many of the alternative splicing events observed in the AD brain (Berson, Barbash et al. 2012). Furthermore knocking down hnRNPA/B proteins produced memory and electrophysiological impairments in the mice (Berson, Barbash et al. 2012). Taken together, these results implicate broad changes in alternative splicing as a causal factor

to cognitive impairments in AD. Importantly, this change in hnRNPA/B protein levels was independent of Aβ and Tau toxicity in mouse models of AD. Rather, it was shown that cholinergic signalling mediated hnRNPA/B protein levels (Berson, Barbash et al. 2012), and that manipulating cholinergic tone in mice could impair RNA metabolism.

An unbiased analysis of the human brain-insoluble proteome identified a number of protein components of the U1-snRNP, both the U1-70K and the U1A proteins, as being aggregated in the AD brain (Bai, Hales et al. 2013). This complex is a primary component of the spliceosome and mediates spliceosome binding to RNA and the initiation of splicing (Hodnett and Busch 1968, Seraphin and Rosbash 1989). In line with the observation of aggregation of splicing factors in the AD brain, a number of genes were shown to be alternatively spiced in the AD brain, including the BACE1 gene (Bai, Hales et al. 2013). In vitro manipulations of U1-70K expression altered APP processing in cultured neurons and induced production of Aβ species (Bai, Hales et al. 2013). Experimental evidence has shown that he aberrant aggregation of the U1-70K splicing factor in the AD brain is the result of abnormal truncation of the protein in the AD brain (Bai, Chen et al. 2014).

Taken together, these findings demonstrate multiple neurodegeneration-related alterations in RNA processing which may play a role in AD pathogenesis and disease progression. The mechanisms regulating these disease related changes in RNA-metabolism remain poorly understood, however cholinergic signalling has been demonstrated as an important mediator of this process. Insight into the cellular mechanisms regulating this interaction could provide crucial insight into the pathogenesis of AD.

1.3.4.3 RNA Sequencing as a Tool to study RNA Metabolism

The study of entire transcriptome at once (the sum of all transcripts within a cell, or group of cells) has proven to be an invaluable tool in neuroscience from basic science (Okaty, Sugino et al. 2011), up to clinical research (Gould and Manji 2004). A number of techniques have been developed in order to study the transcriptome. Sequencing based

approaches is one of the powerful tools used to study of RNA metabolism. The depection of the typical workflow of an RNA-Seq experiment can be found in Figure 1.5.

RNA-Seq can provide an in depth qualitative and quantitative analysis of the transcriptome. The first step in RNA-Seq is the isolation of total RNA from the cell population/tissue of interest. This total RNA is then converted into a library of cDNA and fragmented. Adapter sequences are added to each fragment of cDNA. The adapter sequences provide necessary elements for sequencing and also contain DNA barcodes which allows for the determination of the 5'-3' orientation of the sequence fragment. The cDNA fragments are then sequenced in a high throughput manner, producing "reads" of the fragments. After being sequenced, the resulting "reads" are then aligned using assembly algorithms either with or without a reference genome (Mortazavi, Williams et al. 2008). This mapping process provides the qualitative analysis of the transcriptome, while the quantitative aspect of RNA-Seq comes from the count of the reads from same transcript (Kanitz, Gypas et al. 2015, Conesa, Madrigal et al. 2016). This "reads count" can be used to compare the level of transcripts across experimental conditions.

Given that RNA-Seq can provide quantitative and qualitative analysis of transcripts at an extremely high level of resolution, the technique is an invaluable tool to the study of alternative splicing and RNA processing. The resulting data from RNA-Seq experiments can be analyzed in a number of ways to quantify alternative splicing. The most conservative approach to this kind of analysis is to quantify the number of reads which map to particular transcript isoform which have unique sequences, be they exons, intron or splice junctions (Filichkin, Priest et al. 2010). The normalized count of these reads provides an absolute quantification of the abundance of these transcript variants. This approach however is limited to transcripts which have these unique sequences, or which only have very minor (a couple of base pairs) differences between transcript variants. An alternative approach is to quantify all the reads which map onto a specific gene of interest, and normalizing the reads to the average for that gene. This approach will thus provide the relative abundance of transcript variants, and has led to the discovery of many novel alternative splicing events and splice junctions (Trapnell, Pachter et al. 2009).

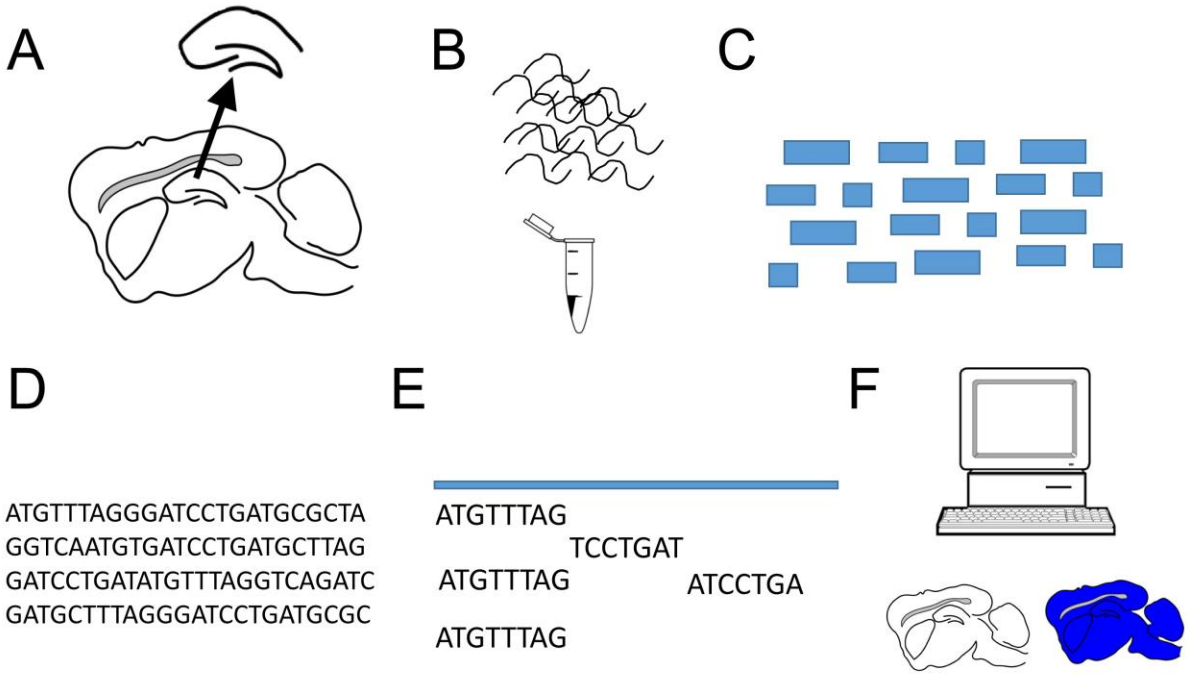


Figure 1.5 Workflow of an RNA-Seq Experiment. (A) A tissue sample of interest is isolated. (B) mRNA is isolated from the tissue sample. (C) The mRNA is converted into cDNA and fragmented into short segments (reads). (D) The reads are sequenced. (E) Sequenced reads are aligned to a reference genome. (F) Aligned sequences are then counted (read counts) to allow for comparisons between samples.

1.4 Rationale and Hypothesis

AD is a progressive neurodegenerative disease and is characterized by a number of key pathological and molecular changes in the brain. These molecular and pathological changes converge to perturb neuronal function, leading to cognitive decline, and ultimately lead to loss of neurons in the brain. A key aspect of AD is the loss of basal forebrain cholinergic neurons. ACh has been implicated in the underlying physiology of many distinct cognitive functions; however the exact role that ACh plays in regulating information processing in the brain is still not fully understood.

Therefore, the overall objective of thesis is to first characterize how altered cholinergic signalling in mice can lead to cognitive dysfunction. Additional objectives include to evaluate the molecular changes that follow long-term cholinergic dysfunction and how they may be related to AD pathology. VAChT is required for the storage of ACh into synaptic vesicles, and therefore it presents a means to modulate release. We can leverage this knowledge to generate mice with brain region specific changes in transporter level. Specifically, the aims of this thesis are:

1. To investigate the consequences of cholinergic dysfunction on distinct cognitive domains in mice and attempt to dissect underlying receptor signalling pathways.
2. Determine the genome-wide transcriptome level changes in mice with long-term cholinergic dysfunction and to determine the molecular mechanisms underpinning these changes.
3. Examine the molecular alterations that contribute to molecular pathology in a mouse model with deficient cholinergic signaling in the forebrain.

Several lines of research suggest that long-term cholinergic dysfunction may lead to cognition dysfunction, suggesting a role for decreased ACh signaling on the broad molecular and pathological changes in the demented brain. *The hypothesis we tested is that changes in cholinergic tone produce specific molecular signatures in target brain areas that underlie alterations in cognitive function.* We combined behavioural and molecular techniques and unique mouse models to test this hypothesis and to define

basic aspects of regulation of behaviour by cholinergic activity. Dysfunction of the cholinergic system has been shown to play a role in a host of neurological and neuropsychiatric disorders beyond just AD. Thus, understanding the basic physiological aspects of this system may lead to the development of novel treatments for neurological and psychiatric disorders.

References

Abbott, L. F. and S. B. Nelson (2000). "Synaptic plasticity: taming the beast." Nat Neurosci **3 Suppl**: 1178-1183.

Abraha, A., N. Ghoshal, T. C. Gamblin, V. Cryns, R. W. Berry, J. Kuret and L. I. Binder (2000). "C-terminal inhibition of tau assembly in vitro and in Alzheimer's disease." J Cell Sci **113 Pt 21**: 3737-3745.

Ahmadian, S. S., A. Rezvanian, M. Peterson, S. Weintraub, E. H. Bigio, M. M. Mesulam and C. Geula (2015). "Loss of calbindin-D28K is associated with the full range of tangle pathology within basal forebrain cholinergic neurons in Alzheimer's disease." Neurobiol Aging **36**(12): 3163-3170.

Ahmed, M., J. Davis, D. Aucoin, T. Sato, S. Ahuja, S. Aimoto, J. I. Elliott, W. E. Van Nostrand and S. O. Smith (2010). "Structural conversion of neurotoxic amyloid-beta(1-42) oligomers to fibrils." Nat Struct Mol Biol **17**(5): 561-567.

Al-Onaizi, M. A., G. M. Parfitt, B. Kolisnyk, C. S. Law, M. S. Guzman, D. M. Barros, L. S. Leung, M. A. Prado and V. F. Prado (2016). "Regulation of Cognitive Processing by Hippocampal Cholinergic Tone." Cereb Cortex.

Alfonso, A., K. Grundahl, J. S. Duerr, H. P. Han and J. B. Rand (1993). "The Caenorhabditis elegans unc-17 gene: a putative vesicular acetylcholine transporter." Science **261**(5121): 617-619.

Alfonso, A., K. Grundahl, J. R. McManus, J. M. Asbury and J. B. Rand (1994). "Alternative splicing leads to two cholinergic proteins in Caenorhabditis elegans." J Mol Biol **241**(4): 627-630.

Alkondon, M. and E. X. Albuquerque (2005). "Nicotinic receptor subtypes in rat hippocampal slices are differentially sensitive to desensitization and early in vivo functional up-regulation by nicotine and to block by bupropion." J Pharmacol Exp Ther **313**(2): 740-750.

Alkondon, M., S. Reinhardt, C. Lobron, B. Hermsen, A. Maelicke and E. X. Albuquerque (1994). "Diversity of nicotinic acetylcholine receptors in rat hippocampal neurons. II. The rundown and inward rectification of agonist-elicited whole-cell currents and identification of receptor subunits by in situ hybridization." J Pharmacol Exp Ther **271**(1): 494-506.

Allen, T. G., F. C. Abogadie and D. A. Brown (2006). "Simultaneous release of glutamate and acetylcholine from single magnocellular "cholinergic" basal forebrain neurons." J Neurosci **26**(5): 1588-1595.

Allgaier, C., B. Daschmann, H. Y. Huang and G. Hertting (1988). "Protein kinase C and presynaptic modulation of acetylcholine release in rabbit hippocampus." Br J Pharmacol **93**(3): 525-534.

Alonso Adel, C., A. Mederlyova, M. Novak, I. Grundke-Iqbal and K. Iqbal (2004). "Promotion of hyperphosphorylation by frontotemporal dementia tau mutations." J Biol Chem **279**(33): 34873-34881.

Anagnostaras, S. G., G. G. Murphy, S. E. Hamilton, S. L. Mitchell, N. P. Rahnama, N. M. Nathanson and A. J. Silva (2003). "Selective cognitive dysfunction in acetylcholine M1 muscarinic receptor mutant mice." Nat Neurosci **6**(1): 51-58.

Anderson, D. C., S. C. King and S. M. Parsons (1983). "Pharmacological characterization of the acetylcholine transport system in purified Torpedo electric organ synaptic vesicles." Mol Pharmacol **24**(1): 48-54.

Aosaki, T. and Y. Kawaguchi (1996). "Actions of substance P on rat neostriatal neurons in vitro." J Neurosci **16**(16): 5141-5153.

Aosaki, T., K. Kiuchi and Y. Kawaguchi (1998). "Dopamine D1-like receptor activation excites rat striatal large aspiny neurons in vitro." J Neurosci **18**(14): 5180-5190.

Aosaki, T., H. Tsubokawa, A. Ishida, K. Watanabe, A. M. Graybiel and M. Kimura (1994). "Responses of tonically active neurons in the primate's striatum undergo systematic changes during behavioral sensorimotor conditioning." J Neurosci **14**(6): 3969-3984.

Apparsundaram, S., S. M. Ferguson and R. D. Blakely (2001). "Molecular cloning and characterization of a murine hemicholinium-3-sensitive choline transporter." Biochem Soc Trans **29**(Pt 6): 711-716.

Apparsundaram, S., S. M. Ferguson, A. L. George, Jr. and R. D. Blakely (2000). "Molecular cloning of a human, hemicholinium-3-sensitive choline transporter." Biochem Biophys Res Commun **276**(3): 862-867.

Apparsundaram, S., V. Martinez, V. Parikh, R. Kozak and M. Sarter (2005). "Increased capacity and density of choline transporters situated in synaptic membranes of the right

medial prefrontal cortex of attentional task-performing rats." J Neurosci **25**(15): 3851-3856.

Atri, A., S. Sherman, K. A. Norman, B. A. Kirchhoff, M. M. Nicolas, M. D. Greicius, S. C. Cramer, H. C. Breiter, M. E. Hasselmo and C. E. Stern (2004). "Blockade of central cholinergic receptors impairs new learning and increases proactive interference in a word paired-associate memory task." Behav Neurosci **118**(1): 223-236.

Auerbach, J. M. and M. Segal (1996). "Muscarinic receptors mediating depression and long-term potentiation in rat hippocampus." J Physiol **492 (Pt 2)**: 479-493.

Bacman, S., L. Sterin-Borda, J. J. Camusso, R. Arana, O. Hubscher and E. Borda (1996). "Circulating antibodies against rat parotid gland M3 muscarinic receptors in primary Sjogren's syndrome." Clin Exp Immunol **104**(3): 454-459.

Bai, B., P. C. Chen, C. M. Hales, Z. Wu, V. Pagala, A. A. High, A. I. Levey, J. J. Lah and J. Peng (2014). "Integrated approaches for analyzing U1-70K cleavage in Alzheimer's disease." J Proteome Res **13**(11): 4526-4534.

Bai, B., C. M. Hales, P. C. Chen, Y. Gozal, E. B. Dammer, J. J. Fritz, X. Wang, Q. Xia, D. M. Duong, C. Street, G. Cantero, D. Cheng, D. R. Jones, Z. Wu, Y. Li, I. Diner, C. J. Heilman, H. D. Rees, H. Wu, L. Lin, K. E. Szulwach, M. Gearing, E. J. Mufson, D. A. Bennett, T. J. Montine, N. T. Seyfried, T. S. Wingo, Y. E. Sun, P. Jin, J. Hanfelt, D. M. Willcock, A. Levey, J. J. Lah and J. Peng (2013). "U1 small nuclear ribonucleoprotein complex and RNA splicing alterations in Alzheimer's disease." Proc Natl Acad Sci U S A **110**(41): 16562-16567.

Baker-Nigh, A., S. Vahedi, E. G. Davis, S. Weintraub, E. H. Bigio, W. L. Klein and C. Geula (2015). "Neuronal amyloid-beta accumulation within cholinergic basal forebrain in ageing and Alzheimer's disease." Brain **138**(Pt 6): 1722-1737.

Bandyopadhyay, S., L. E. Goldstein, D. K. Lahiri and J. T. Rogers (2007). "Role of the APP non-amyloidogenic signaling pathway and targeting alpha-secretase as an alternative drug target for treatment of Alzheimer's disease." Curr Med Chem **14**(27): 2848-2864.

Barbosa, J., Jr., A. D. Clarizia, M. V. Gomez, M. A. Romano-Silva, V. F. Prado and M. A. Prado (1997). "Effect of protein kinase C activation on the release of [3H]acetylcholine in the presence of vesamicol." J Neurochem **69**(6): 2608-2611.

Barbosa, J., Jr., L. T. Ferreira, C. Martins-Silva, M. S. Santos, G. E. Torres, M. G. Caron, M. V. Gomez, S. S. Ferguson, M. A. Prado and V. F. Prado (2002). "Trafficking of the vesicular acetylcholine transporter in SN56 cells: a dynamin-sensitive step and interaction with the AP-2 adaptor complex." J Neurochem **82**(5): 1221-1228.

Barbosa, J., Jr., A. R. Massensini, M. S. Santos, S. I. Meireles, R. S. Gomez, M. V. Gomez, M. A. Romano-Silva, V. F. Prado and M. A. Prado (1999). "Expression of the vesicular acetylcholine transporter, proteins involved in exocytosis, and functional calcium signaling in varicosities and soma of a murine septal cell line." J Neurochem **73**(5): 1881-1893.

Bartko, S. J., C. Romberg, B. White, J. Wess, T. J. Bussey and L. M. Saksida (2011). "Intact attentional processing but abnormal responding in M1 muscarinic receptor-deficient mice using an automated touchscreen method." Neuropharmacology **61**(8): 1366-1378.

Bartus, R. T., R. L. Dean, 3rd, B. Beer and A. S. Lippa (1982). "The cholinergic hypothesis of geriatric memory dysfunction." Science **217**(4558): 408-414.

Basile, A. S., I. Fedorova, A. Zapata, X. Liu, T. Shippenberg, A. Duttaroy, M. Yamada and J. Wess (2002). "Deletion of the M5 muscarinic acetylcholine receptor attenuates morphine reinforcement and withdrawal but not morphine analgesia." Proc Natl Acad Sci U S A **99**(17): 11452-11457.

Baumann, K., E. M. Mandelkow, J. Biernat, H. Piwnica-Worms and E. Mandelkow (1993). "Abnormal Alzheimer-like phosphorylation of tau-protein by cyclin-dependent kinases cdk2 and cdk5." FEBS Lett **336**(3): 417-424.

Baxter, M. G., D. J. Bucci, L. K. Gorman, R. G. Wiley and M. Gallagher (2013). "Selective immunotoxic lesions of basal forebrain cholinergic cells: effects on learning and memory in rats." Behav Neurosci **127**(5): 619-627.

Bear, M. F. and W. Singer (1986). "Modulation of visual cortical plasticity by acetylcholine and noradrenaline." Nature **320**(6058): 172-176.

Bejanin, S., R. Cervini, J. Mallet and S. Berrard (1994). "A unique gene organization for two cholinergic markers, choline acetyltransferase and a putative vesicular transporter of acetylcholine." J Biol Chem **269**(35): 21944-21947.

Bejanin, S., E. Habert, S. Berrard, J. B. Edwards, J. P. Loeffler and J. Mallet (1992). "Promoter elements of the rat choline acetyltransferase gene allowing nerve growth factor inducibility in transfected primary cultured cells." J Neurochem **58**(4): 1580-1583.

Bell, M. I., P. J. Richardson and K. Lee (2000). "Histamine depolarizes cholinergic interneurones in the rat striatum via a H(1)-receptor mediated action." Br J Pharmacol **131**(6): 1135-1142.

Benarroch, E. E. (2013). "Pedunculopontine nucleus: functional organization and clinical implications." Neurology **80**(12): 1148-1155.

Benchenane, K., P. H. Tiesinga and F. P. Battaglia (2011). "Oscillations in the prefrontal cortex: a gateway to memory and attention." Curr Opin Neurobiol **21**(3): 475-485.

Berkeley, J. L., J. Gomeza, J. Wess, S. E. Hamilton, N. M. Nathanson and A. I. Levey (2001). "M1 muscarinic acetylcholine receptors activate extracellular signal-regulated kinase in CA1 pyramidal neurons in mouse hippocampal slices." Mol Cell Neurosci **18**(5): 512-524.

Berrard, S., A. Brice, F. Lottspeich, A. Braun, Y. A. Barde and J. Mallet (1987). "cDNA cloning and complete sequence of porcine choline acetyltransferase: in vitro translation of the corresponding RNA yields an active protein." Proc Natl Acad Sci U S A **84**(24): 9280-9284.

Berrard, S., H. Varoqui, R. Cervini, M. Israel, J. Mallet and M. F. Diebler (1995). "Coregulation of two embedded gene products, choline acetyltransferase and the vesicular acetylcholine transporter." J Neurochem **65**(2): 939-942.

Berson, A., S. Barbash, G. Shaltiel, Y. Goll, G. Hanin, D. S. Greenberg, M. Ketzef, A. J. Becker, A. Friedman and H. Soreq (2012). "Cholinergic-associated loss of hnRNP-A/B in Alzheimer's disease impairs cortical splicing and cognitive function in mice." EMBO Mol Med **4**(8): 730-742.

Berstein, G., J. L. Blank, A. V. Smrcka, T. Higashijima, P. C. Sternweis, J. H. Exton and E. M. Ross (1992). "Reconstitution of agonist-stimulated phosphatidylinositol 4,5-bisphosphate hydrolysis using purified m1 muscarinic receptor, Gq/11, and phospholipase C-beta 1." J Biol Chem **267**(12): 8081-8088.

Beyer, A. L., M. E. Christensen, B. W. Walker and W. M. LeStourgeon (1977). "Identification and characterization of the packaging proteins of core 40S hnRNP particles." Cell **11**(1): 127-138.

Birks, R. I. and F. C. Macintosh (1957). "Acetylcholine metabolism at nerve-endings." Br Med Bull **13**(3): 157-161.

Birks, R. I., F. C. Macintosh and P. B. Sastry (1956). "Pharmacological inhibition of acetylcholine synthesis." Nature **178**(4543): 1181.

Birks, R. I., K. J. Worsley and R. I. Birks (1985). "Activation of acetylcholine synthesis in cat sympathetic ganglia: dependence on external choline and sodium-pump rate." J Physiol **367**: 401-417.

Birrell, J. M. and V. J. Brown (2000). "Medial frontal cortex mediates perceptual attentional set shifting in the rat." J Neurosci **20**(11): 4320-4324.

Black, D. L. (2003). "Mechanisms of alternative pre-messenger RNA splicing." Annu Rev Biochem **72**: 291-336.

Blencowe, B. J. (2000). "Exonic splicing enhancers: mechanism of action, diversity and role in human genetic diseases." Trends Biochem Sci **25**(3): 106-110.

Boegman, R. J., J. Cockhill, K. Jhamandas and R. J. Beninger (1992). "Excitotoxic lesions of rat basal forebrain: differential effects on choline acetyltransferase in the cortex and amygdala." Neuroscience **51**(1): 129-135.

Bolam, J. P., B. H. Wainer and A. D. Smith (1984). "Characterization of cholinergic neurons in the rat neostriatum. A combination of choline acetyltransferase immunocytochemistry, Golgi-impregnation and electron microscopy." Neuroscience **12**(3): 711-718.

Bonifaci, N., J. Moroianu, A. Radu and G. Blobel (1997). "Karyopherin beta2 mediates nuclear import of a mRNA binding protein." Proc Natl Acad Sci U S A **94**(10): 5055-5060.

Bonsi, P., D. Cuomo, J. Ding, G. Sciamanna, S. Ulrich, A. Tscherter, G. Bernardi, D. J. Surmeier and A. Pisani (2007). "Endogenous serotonin excites striatal cholinergic interneurons via the activation of 5-HT 2C, 5-HT6, and 5-HT7 serotonin receptors: implications for extrapyramidal side effects of serotonin reuptake inhibitors." Neuropsychopharmacology **32**(8): 1840-1854.

Book, A. A., R. G. Wiley and J. B. Schweitzer (1992). "Specificity of 192 IgG-saporin for NGF receptor-positive cholinergic basal forebrain neurons in the rat." Brain Res **590**(1-2): 350-355.

Bowen, D. M., C. B. Smith, P. White and A. N. Davison (1976). "Neurotransmitter-related enzymes and indices of hypoxia in senile dementia and other abiotrophies." Brain **99**(3): 459-496.

Braak, H., E. Braak, I. Grundke-Iqbal and K. Iqbal (1986). "Occurrence of neuropil threads in the senile human brain and in Alzheimer's disease: a third location of paired helical filaments outside of neurofibrillary tangles and neuritic plaques." Neurosci Lett **65**(3): 351-355.

Brandon, E. P., W. Lin, K. A. D'Amour, D. P. Pizzo, B. Dominguez, Y. Sugiura, S. Thode, C. P. Ko, L. J. Thal, F. H. Gage and K. F. Lee (2003). "Aberrant patterning of neuromuscular synapses in choline acetyltransferase-deficient mice." J Neurosci **23**(2): 539-549.

Brandon, E. P., T. Mellott, D. P. Pizzo, N. Coufal, K. A. D'Amour, K. Gobeske, M. Lortie, I. Lopez-Coviella, B. Berse, L. J. Thal, F. H. Gage and J. K. Blusztajn (2004). "Choline transporter 1 maintains cholinergic function in choline acetyltransferase haploinsufficiency." J Neurosci **24**(24): 5459-5466.

Breitbart, R. E., A. Andreadis and B. Nadal-Ginard (1987). "Alternative splicing: a ubiquitous mechanism for the generation of multiple protein isoforms from single genes." Annu Rev Biochem **56**: 467-495.

Brigman, J. L., R. A. Daut, T. Wright, O. Gunduz-Cinar, C. Graybeal, M. I. Davis, Z. Jiang, L. M. Saksida, S. Jinde, M. Pease, T. J. Bussey, D. M. Lovinger, K. Nakazawa and A. Holmes (2013). "GluN2B in corticostriatal circuits governs choice learning and choice shifting." Nat Neurosci **16**(8): 1101-1110.

Brigman, J. L., M. Feyder, L. M. Saksida, T. J. Bussey, M. Mishina and A. Holmes (2008). "Impaired discrimination learning in mice lacking the NMDA receptor NR2A subunit." Learn Mem **15**(2): 50-54.

Brock, M., A. C. Nickel, B. Madziar, J. K. Blusztajn and B. Berse (2007). "Differential regulation of the high affinity choline transporter and the cholinergic locus by cAMP signaling pathways." Brain Res **1145**: 1-10.

Broussard, J. I., K. Karelina, M. Sarter and B. Givens (2009). "Cholinergic optimization of cue-evoked parietal activity during challenged attentional performance." Eur J Neurosci **29**(8): 1711-1722.

Brown, S. J., S. James, M. Reddington and P. J. Richardson (1990). "Both A1 and A2a purine receptors regulate striatal acetylcholine release." J Neurochem **55**(1): 31-38.

Bruce, G. and L. B. Hersh (1987). "Studies on detergent released choline acetyltransferase from membrane fractions of rat and human brain." Neurochem Res **12**(12): 1059-1066.

Bruce, G. and L. B. Hersh (1989). "The phosphorylation of choline acetyltransferase." Neurochem Res **14**(7): 613-620.

Bruce, G., B. H. Wainer and L. B. Hersh (1985). "Immunoaffinity purification of human choline acetyltransferase: comparison of the brain and placental enzymes." J Neurochem **45**(2): 611-620.

Bueno-Junior, L. S., C. Lopes-Aguiar, R. N. Ruggiero, R. N. Romcy-Pereira and J. P. Leite (2012). "Muscarinic and nicotinic modulation of thalamo-prefrontal cortex synaptic plasticity [corrected] in vivo." PLoS One **7**(10): e47484.

Buhl, E. H., G. Tamas and A. Fisahn (1998). "Cholinergic activation and tonic excitation induce persistent gamma oscillations in mouse somatosensory cortex in vitro." J Physiol **513 (Pt 1)**: 117-126.

Burd, C. G., M. S. Swanson, M. Gorlach and G. Dreyfuss (1989). "Primary structures of the heterogeneous nuclear ribonucleoprotein A2, B1, and C2 proteins: a diversity of RNA binding proteins is generated by small peptide inserts." Proc Natl Acad Sci U S A **86**(24): 9788-9792.

Burk, J. A., C. D. Herzog, M. C. Porter and M. Sarter (2002). "Interactions between aging and cortical cholinergic deafferentation on attention." Neurobiol Aging **23**(3): 467-477.

Caccamo, A., S. Oddo, L. M. Billings, K. N. Green, H. Martinez-Coria, A. Fisher and F. M. LaFerla (2006). "M1 receptors play a central role in modulating AD-like pathology in transgenic mice." Neuron **49**(5): 671-682.

Cachope, R., Y. Mateo, B. N. Mathur, J. Irving, H. L. Wang, M. Morales, D. M. Lovinger and J. F. Cheer (2012). "Selective activation of cholinergic interneurons enhances

accumbal phasic dopamine release: setting the tone for reward processing." Cell Rep **2**(1): 33-41.

Cai, D., J. Y. Leem, J. P. Greenfield, P. Wang, B. S. Kim, R. Wang, K. O. Lopes, S. H. Kim, H. Zheng, P. Greengard, S. S. Sisodia, G. Thinakaran and H. Xu (2003). "Presenilin-1 regulates intracellular trafficking and cell surface delivery of beta-amyloid precursor protein." J Biol Chem **278**(5): 3446-3454.

Cai, H., Y. Wang, D. McCarthy, H. Wen, D. R. Borchelt, D. L. Price and P. C. Wong (2001). "BACE1 is the major beta-secretase for generation of Abeta peptides by neurons." Nat Neurosci **4**(3): 233-234.

Calabresi, P., D. Centonze, P. Gubellini, A. Pisani and G. Bernardi (1998). "Blockade of M2-like muscarinic receptors enhances long-term potentiation at corticostriatal synapses." Eur J Neurosci **10**(9): 3020-3023.

Calabresi, P., D. Centonze, A. Pisani, G. Sancesario, P. Gubellini, G. A. Marfia and G. Bernardi (1998). "Striatal spiny neurons and cholinergic interneurons express differential ionotropic glutamatergic responses and vulnerability: implications for ischemia and Huntington's disease." Ann Neurol **43**(5): 586-597.

Campillos, M., J. R. Lamas, M. A. Garcia, M. J. Bullido, F. Valdivieso and J. Vazquez (2003). "Specific interaction of heterogeneous nuclear ribonucleoprotein A1 with the -219T allelic form modulates APOE promoter activity." Nucleic Acids Res **31**(12): 3063-3070.

Campos, F., M. Alfonso and R. Duran (2010). "In vivo modulation of alpha7 nicotinic receptors on striatal glutamate release induced by anatoxin-A." Neurochem Int **56**(6-7): 850-855.

Carbini, L. A. and L. B. Hersh (1993). "Functional analysis of conserved histidines in choline acetyltransferase by site-directed mutagenesis." J Neurochem **61**(1): 247-253.

Carriere, I., A. Fourrier-Reglat, J. F. Dartigues, O. Rouaud, F. Pasquier, K. Ritchie and M. L. Ancelin (2009). "Drugs with anticholinergic properties, cognitive decline, and dementia in an elderly general population: the 3-city study." Arch Intern Med **169**(14): 1317-1324.

Caruana, D. A., E. C. Warburton and Z. I. Bashir (2011). "Induction of activity-dependent LTD requires muscarinic receptor activation in medial prefrontal cortex." J Neurosci **31**(50): 18464-18478.

Celesia, G. G. and H. H. Jasper (1966). "Acetylcholine released from cerebral cortex in relation to state of activation." Neurology **16**(11): 1053-1063.

Cenini, G., C. Cecchi, A. Pensalfini, S. A. Bonini, G. Ferrari-Toninelli, G. Liguri, M. Memo and D. Uberti (2010). "Generation of reactive oxygen species by beta amyloid fibrils and oligomers involves different intra/extracellular pathways." Amino Acids **38**(4): 1101-1106.

Charara, A., Y. Smith and A. Parent (1996). "Glutamatergic inputs from the pedunculopontine nucleus to midbrain dopaminergic neurons in primates: Phaseolus vulgaris-leucoagglutinin anterograde labeling combined with postembedding glutamate and GABA immunohistochemistry." J Comp Neurol **364**(2): 254-266.

Chen, K. H., E. A. Reese, H. W. Kim, S. I. Rapoport and J. S. Rao (2011). "Disturbed neurotransmitter transporter expression in Alzheimer's disease brain." J Alzheimers Dis **26**(4): 755-766.

Chen, S., S. P. Yadav and W. K. Surewicz (2010). "Interaction between human prion protein and amyloid-beta (Abeta) oligomers: role OF N-terminal residues." J Biol Chem **285**(34): 26377-26383.

Chudasama, Y., J. W. Dalley, F. Nathwani, P. Bouger and T. W. Robbins (2004). "Cholinergic modulation of visual attention and working memory: dissociable effects of basal forebrain 192-IgG-saporin lesions and intraprefrontal infusions of scopolamine." Learn Mem **11**(1): 78-86.

Chuhma, N., S. Mingote, H. Moore and S. Rayport (2014). "Dopamine neurons control striatal cholinergic neurons via regionally heterogeneous dopamine and glutamate signaling." Neuron **81**(4): 901-912.

Chuhma, N., K. F. Tanaka, R. Hen and S. Rayport (2011). "Functional connectome of the striatal medium spiny neuron." J Neurosci **31**(4): 1183-1192.

Chui, D. H., H. Tanahashi, K. Ozawa, S. Ikeda, F. Checler, O. Ueda, H. Suzuki, W. Araki, H. Inoue, K. Shirotani, K. Takahashi, F. Gallyas and T. Tabira (1999).

"Transgenic mice with Alzheimer presenilin 1 mutations show accelerated neurodegeneration without amyloid plaque formation." Nat Med **5**(5): 560-564.
Citron, M., D. Westaway, W. Xia, G. Carlson, T. Diehl, G. Levesque, K. Johnson-Wood, M. Lee, P. Seubert, A. Davis, D. Kholodenko, R. Motter, R. Sherrington, B. Perry, H. Yao, R. Strome, I. Lieberburg, J. Rommens, S. Kim, D. Schenk, P. Fraser, P. St George Hyslop and D. J. Selkoe (1997). "Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid beta-protein in both transfected cells and transgenic mice." Nat Med **3**(1): 67-72.
Clark, L., R. Cools and T. W. Robbins (2004). "The neuropsychology of ventral prefrontal cortex: decision-making and reversal learning." Brain Cogn **55**(1): 41-53.
Cleary, J. P., D. M. Walsh, J. J. Hofmeister, G. M. Shankar, M. A. Kuskowski, D. J. Selkoe and K. H. Ashe (2005). "Natural oligomers of the amyloid-beta protein specifically disrupt cognitive function." Nat Neurosci **8**(1): 79-84.
Collier, B. and H. S. Katz (1974). "Acetylcholine synthesis from recaptured choline by a sympathetic ganglion." J Physiol **238**(3): 639-655.
Collier, B., S. A. Welner, J. Ricny and D. M. Araujo (1986). "Acetylcholine synthesis and release by a sympathetic ganglion in the presence of 2-(4-phenylpiperidino) cyclohexanol (AH5183)." J Neurochem **46**(3): 822-830.
Collingridge, G. L., S. Peineau, J. G. Howland and Y. T. Wang (2010). "Long-term depression in the CNS." Nat Rev Neurosci **11**(7): 459-473.
Conesa, A., P. Madrigal, S. Tarazona, D. Gomez-Cabrero, A. Cervera, A. McPherson, M. W. Szczesniak, D. J. Gaffney, L. L. Elo, X. Zhang and A. Mortazavi (2016). "A survey of best practices for RNA-seq data analysis." Genome Biol **17**(1): 13.
Conner, J. M., A. Culberson, C. Packowski, A. A. Chiba and M. H. Tuszynski (2003). "Lesions of the Basal forebrain cholinergic system impair task acquisition and abolish cortical plasticity associated with motor skill learning." Neuron **38**(5): 819-829.
Corrigall, W. A., K. M. Coen, J. Zhang and L. Adamson (2002). "Pharmacological manipulations of the pedunculopontine tegmental nucleus in the rat reduce self-administration of both nicotine and cocaine." Psychopharmacology (Berl) **160**(2): 198-205.

Couey, J. J., R. M. Meredith, S. Spijker, R. B. Poorthuis, A. B. Smit, A. B. Brussaard and H. D. Mansvelder (2007). "Distributed network actions by nicotine increase the threshold for spike-timing-dependent plasticity in prefrontal cortex." Neuron **54**(1): 73-87.

Cramer, P., J. F. Caceres, D. Cazalla, S. Kadener, A. F. Muro, F. E. Baralle and A. R. Kornblihtt (1999). "Coupling of transcription with alternative splicing: RNA pol II promoters modulate SF2/ASF and 9G8 effects on an exonic splicing enhancer." Mol Cell **4**(2): 251-258.

Cuello, A. C. and M. A. Bruno (2007). "The failure in NGF maturation and its increased degradation as the probable cause for the vulnerability of cholinergic neurons in Alzheimer's disease." Neurochem Res **32**(6): 1041-1045.

Cummings, J. L. (2003). "Use of cholinesterase inhibitors in clinical practice: evidence-based recommendations." Am J Geriatr Psychiatry **11**(2): 131-145.

Dalley, J. W., J. McGaughy, M. T. O'Connell, R. N. Cardinal, L. Levita and T. W. Robbins (2001). "Distinct changes in cortical acetylcholine and noradrenaline efflux during contingent and noncontingent performance of a visual attentional task." J Neurosci **21**(13): 4908-4914.

Dani, J. A. and D. Bertrand (2007). "Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system." Annu Rev Pharmacol Toxicol **47**: 699-729.

Dautan, D., I. Huerta-Ocampo, I. B. Witten, K. Deisseroth, J. P. Bolam, T. Gerdjikov and J. Mena-Segovia (2014). "A major external source of cholinergic innervation of the striatum and nucleus accumbens originates in the brainstem." J Neurosci **34**(13): 4509-4518.

Davidson, M. C. and R. T. Marrocco (2000). "Local infusion of scopolamine into intraparietal cortex slows covert orienting in rhesus monkeys." J Neurophysiol **83**(3): 1536-1549.

Davies, P. and A. J. Maloney (1976). "Selective loss of central cholinergic neurons in Alzheimer's disease." Lancet **2**(8000): 1403.

Davis, A. A., J. J. Fritz, J. Wess, J. J. Lah and A. I. Levey (2010). "Deletion of M1 muscarinic acetylcholine receptors increases amyloid pathology in vitro and in vivo." J Neurosci **30**(12): 4190-4196.

Davis, K. L., R. C. Mohs, D. Marin, D. P. Purohit, D. P. Perl, M. Lantz, G. Austin and V. Haroutunian (1999). "Cholinergic markers in elderly patients with early signs of Alzheimer disease." JAMA **281**(15): 1401-1406.

de Bruin, J. P., F. Sanchez-Santed, R. P. Heinsbroek, A. Donker and P. Postmes (1994). "A behavioural analysis of rats with damage to the medial prefrontal cortex using the Morris water maze: evidence for behavioural flexibility, but not for impaired spatial navigation." Brain Res **652**(2): 323-333.

de Castro, B. M., X. De Jaeger, C. Martins-Silva, R. D. Lima, E. Amaral, C. Menezes, P. Lima, C. M. Neves, R. G. Pires, T. W. Gould, I. Welch, C. Kushmerick, C. Guatimosim, I. Izquierdo, M. Cammarota, R. J. Rylett, M. V. Gomez, M. G. Caron, R. W. Oppenheim, M. A. Prado and V. F. Prado (2009). "The vesicular acetylcholine transporter is required for neuromuscular development and function." Mol Cell Biol **29**(19): 5238-5250.

De Felice, F. G. and S. T. Ferreira (2002). "Beta-amyloid production, aggregation, and clearance as targets for therapy in Alzheimer's disease." Cell Mol Neurobiol **22**(5-6): 545-563.

de Jonge, W. J. and L. Ulloa (2007). "The alpha7 nicotinic acetylcholine receptor as a pharmacological target for inflammation." Br J Pharmacol **151**(7): 915-929.

De Jonghe, C., C. Esselens, S. Kumar-Singh, K. Craessaerts, S. Serneels, F. Checler, W. Annaert, C. Van Broeckhoven and B. De Strooper (2001). "Pathogenic APP mutations near the gamma-secretase cleavage site differentially affect Abeta secretion and APP C-terminal fragment stability." Hum Mol Genet **10**(16): 1665-1671.

De Leon, M. J., A. E. George, J. Golomb, C. Tarshish, A. Convit, A. Kluger, S. De Santi, T. McRae, S. H. Ferris, B. Reisberg, C. Ince, H. Rusinek, M. Bobinski, B. Quinn, D. C. Miller and H. M. Wisniewski (1997). "Frequency of hippocampal formation atrophy in normal aging and Alzheimer's disease." Neurobiol Aging **18**(1): 1-11.

De Strooper, B., W. Annaert, P. Cupers, P. Saftig, K. Craessaerts, J. S. Mumm, E. H. Schroeter, V. Schrijvers, M. S. Wolfe, W. J. Ray, A. Goate and R. Kopan (1999). "A

presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain." Nature **398**(6727): 518-522.

DeBoer, P. and B. H. Westerink (1994). "GABAergic modulation of striatal cholinergic interneurons: an in vivo microdialysis study." J Neurochem **62**(1): 70-75.

DeKosky, S. T., M. D. Ikonomovic, S. D. Styren, L. Beckett, S. Wisniewski, D. A. Bennett, E. J. Cochran, J. H. Kordower and E. J. Mufson (2002). "Upregulation of choline acetyltransferase activity in hippocampus and frontal cortex of elderly subjects with mild cognitive impairment." Ann Neurol **51**(2): 145-155.

DeKosky, S. T. and S. W. Scheff (1990). "Synapse loss in frontal cortex biopsies in Alzheimer's disease: correlation with cognitive severity." Ann Neurol **27**(5): 457-464.

Delatour, B. and P. Gisquet-Verrier (2000). "Functional role of rat prelimbic-infralimbic cortices in spatial memory: evidence for their involvement in attention and behavioural flexibility." Behav Brain Res **109**(1): 113-128.

Demuro, A., E. Mina, R. Kayed, S. C. Milton, I. Parker and C. G. Glabe (2005). "Calcium dysregulation and membrane disruption as a ubiquitous neurotoxic mechanism of soluble amyloid oligomers." J Biol Chem **280**(17): 17294-17300.

Deutsch, J. A. (1971). "The cholinergic synapse and the site of memory." Science **174**(4011): 788-794.

Dias, R. and J. P. Aggleton (2000). "Effects of selective excitotoxic prefrontal lesions on acquisition of nonmatching- and matching-to-place in the T-maze in the rat: differential involvement of the prelimbic-infralimbic and anterior cingulate cortices in providing behavioural flexibility." Eur J Neurosci **12**(12): 4457-4466.

Dobransky, T., W. L. Davis, G. H. Xiao and R. J. Rylett (2000). "Expression, purification and characterization of recombinant human choline acetyltransferase: phosphorylation of the enzyme regulates catalytic activity." Biochem J **349**(Pt 1): 141-151.

Dobransky, T., A. Doherty-Kirby, A. R. Kim, D. Brewer, G. Lajoie and R. J. Rylett (2004). "Protein kinase C isoforms differentially phosphorylate human choline acetyltransferase regulating its catalytic activity." J Biol Chem **279**(50): 52059-52068.

Dobransky, T. and R. J. Rylett (2003). "Functional regulation of choline acetyltransferase by phosphorylation." Neurochem Res **28**(3-4): 537-542.

Dobransky, T. and R. J. Rylett (2005). "A model for dynamic regulation of choline acetyltransferase by phosphorylation." J Neurochem **95**(2): 305-313.

Docherty, M., H. F. Bradford, J. Y. Wu, T. H. Joh and D. J. Reis (1985). "Evidence for specific immunolysis of nerve terminals using antisera against choline acetyltransferase, glutamate decarboxylase and tyrosine hydroxylase." Brain Res **339**(1): 105-113.

Doebler, J. A., W. R. Markesbery, A. Anthony and R. E. Rhoads (1987). "Neuronal RNA in relation to neuronal loss and neurofibrillary pathology in the hippocampus in Alzheimer's disease." J Neuropathol Exp Neurol **46**(1): 28-39.

Doralp, S. and L. S. Leung (2008). "Cholinergic modulation of hippocampal CA1 basal-dendritic long-term potentiation." Neurobiol Learn Mem **90**(2): 382-388.

Dovey, H. F., V. John, J. P. Anderson, L. Z. Chen, P. de Saint Andrieu, L. Y. Fang, S. B. Freedman, B. Folmer, E. Goldbach, E. J. Holsztynska, K. L. Hu, K. L. Johnson-Wood, S. L. Kennedy, D. Kholodenko, J. E. Knops, L. H. Latimer, M. Lee, Z. Liao, I. M. Lieberburg, R. N. Motter, L. C. Mutter, J. Nietz, K. P. Quinn, K. L. Sacchi, P. A. Seubert, G. M. Shopp, E. D. Thorsett, J. S. Tung, J. Wu, S. Yang, C. T. Yin, D. B. Schenk, P. C. May, L. D. Altstiel, M. H. Bender, L. N. Boggs, T. C. Britton, J. C. Clemens, D. L. Czilli, D. K. Dieckman-McGinty, J. J. Droste, K. S. Fuson, B. D. Gitter, P. A. Hyslop, E. M. Johnstone, W. Y. Li, S. P. Little, T. E. Mabry, F. D. Miller and J. E. Audia (2001). "Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in brain." J Neurochem **76**(1): 173-181.

Drever, B. D., G. Riedel and B. Platt (2011). "The cholinergic system and hippocampal plasticity." Behav Brain Res **221**(2): 505-514.

Dreyfuss, G., M. J. Matunis, S. Pinol-Roma and C. G. Burd (1993). "hnRNP proteins and the biogenesis of mRNA." Annu Rev Biochem **62**: 289-321.

Dunne, M. P. and L. R. Hartley (1986). "Scopolamine and the control of attention in humans." Psychopharmacology (Berl) **89**(1): 94-97.

Dunnett, S. B. and S. D. Iversen (1981). "Learning impairments following selective kainic acid-induced lesions within the neostriatum of rats." Behav Brain Res **2**(2): 189-209.

Durkin, T. P. (1994). "Spatial working memory over long retention intervals: dependence on sustained cholinergic activation in the septohippocampal or nucleus basalis magnocellularis-cortical pathways?" Neuroscience **62**(3): 681-693.

Durkin, T. P. and A. Toumane (1992). "Septo-hippocampal and nBM-cortical cholinergic neurones exhibit differential time-courses of activation as a function of both type and duration of spatial memory testing in mice." Behav Brain Res **50**(1-2): 43-52.

Eckenstein, F. and H. Thoenen (1982). "Production of specific antisera and monoclonal antibodies to choline acetyltransferase: characterization and use for identification of cholinergic neurons." EMBO J **1**(3): 363-368.

Edbauer, D., E. Winkler, J. T. Regula, B. Pesold, H. Steiner and C. Haass (2003). "Reconstitution of gamma-secretase activity." Nat Cell Biol **5**(5): 486-488.

Efange, S. M., E. M. Garland, J. K. Staley, A. B. Khare and D. C. Mash (1997). "Vesicular acetylcholine transporter density and Alzheimer's disease." Neurobiol Aging **18**(4): 407-413.

Eiden, L. E. (1998). "The cholinergic gene locus." J Neurochem **70**(6): 2227-2240.

El Mestikawy, S., A. Wallen-Mackenzie, G. M. Fortin, L. Descarries and L. E. Trudeau (2011). "From glutamate co-release to vesicular synergy: vesicular glutamate transporters." Nat Rev Neurosci **12**(4): 204-216.

English, B. A., M. K. Hahn, I. R. Gizer, M. Mazei-Robison, A. Steele, D. M. Kurnik, M. A. Stein, I. D. Waldman and R. D. Blakely (2009). "Choline transporter gene variation is associated with attention-deficit hyperactivity disorder." J Neurodev Disord **1**(4): 252-263.

Ennulat, D. J., R. K. Liem, G. A. Hashim and M. L. Shelanski (1989). "Two separate 18-amino acid domains of tau promote the polymerization of tubulin." J Biol Chem **264**(10): 5327-5330.

Erickson, J. D., H. Varoqui, M. K. Schafer, W. Modi, M. F. Diebler, E. Weihe, J. Rand, L. E. Eiden, T. I. Bonner and T. B. Usdin (1994). "Functional identification of a vesicular acetylcholine transporter and its expression from a "cholinergic" gene locus." J Biol Chem **269**(35): 21929-21932.

Esch, F. S., P. S. Keim, E. C. Beattie, R. W. Blacher, A. R. Culwell, T. Oltersdorf, D. McClure and P. J. Ward (1990). "Cleavage of amyloid beta peptide during constitutive processing of its precursor." Science **248**(4959): 1122-1124.

Fadda, F., F. Melis and R. Stancampiano (1996). "Increased hippocampal acetylcholine release during a working memory task." Eur J Pharmacol **307**(2): R1-2.

Fallon, J. H. and R. Y. Moore (1978). "Catecholamine innervation of the basal forebrain. IV. Topography of the dopamine projection to the basal forebrain and neostriatum." J Comp Neurol **180**(3): 545-580.

Ferguson, S. M., M. Bazalakova, V. Savchenko, J. C. Tapia, J. Wright and R. D. Blakely (2004). "Lethal impairment of cholinergic neurotransmission in hemicholinium-3-sensitive choline transporter knockout mice." Proc Natl Acad Sci U S A **101**(23): 8762-8767.

Ferguson, S. M., V. Savchenko, S. Apparsundaram, M. Zwick, J. Wright, C. J. Heilman, H. Yi, A. I. Levey and R. D. Blakely (2003). "Vesicular localization and activity-dependent trafficking of presynaptic choline transporters." J Neurosci **23**(30): 9697-9709.

Fernandes, C., E. Hoyle, E. Dempster, L. C. Schalkwyk and D. A. Collier (2006). "Performance deficit of alpha7 nicotinic receptor knockout mice in a delayed matching-to-place task suggests a mild impairment of working/episodic-like memory." Genes Brain Behav **5**(6): 433-440.

Ferraiuolo, L., P. R. Heath, H. Holden, P. Kasher, J. Kirby and P. J. Shaw (2007). "Microarray analysis of the cellular pathways involved in the adaptation to and progression of motor neuron injury in the SOD1 G93A mouse model of familial ALS." J Neurosci **27**(34): 9201-9219.

Ferreira, L. T., M. S. Santos, N. G. Kolmakova, J. Koenen, J. Barbosa, Jr., M. V. Gomez, C. Guatimosim, X. Zhang, S. M. Parsons, V. F. Prado and M. A. Prado (2005). "Structural requirements for steady-state localization of the vesicular acetylcholine transporter." J Neurochem **94**(4): 957-969.

Ferrer, I., T. Gomez-Isla, B. Puig, M. Freixes, E. Ribe, E. Dalfo and J. Avila (2005). "Current advances on different kinases involved in tau phosphorylation, and implications in Alzheimer's disease and tauopathies." Curr Alzheimer Res **2**(1): 3-18.

Filichkin, S. A., H. D. Priest, S. A. Givan, R. Shen, D. W. Bryant, S. E. Fox, W. K. Wong and T. C. Mockler (2010). "Genome-wide mapping of alternative splicing in Arabidopsis thaliana." Genome Res **20**(1): 45-58.

Fisahn, A., F. G. Pike, E. H. Buhl and O. Paulsen (1998). "Cholinergic induction of network oscillations at 40 Hz in the hippocampus in vitro." Nature **394**(6689): 186-189.

Fisahn, A., M. Yamada, A. Duttaroy, J. W. Gan, C. X. Deng, C. J. McBain and J. Wess (2002). "Muscarinic induction of hippocampal gamma oscillations requires coupling of the M1 receptor to two mixed cation currents." Neuron **33**(4): 615-624.

Forster, G. L., J. S. Yeomans, J. Takeuchi and C. D. Blaha (2002). "M5 muscarinic receptors are required for prolonged accumbal dopamine release after electrical stimulation of the pons in mice." J Neurosci **22**(1): RC190.

Francis, R., G. McGrath, J. Zhang, D. A. Ruddy, M. Sym, J. Apfeld, M. Nicoll, M. Maxwell, B. Hai, M. C. Ellis, A. L. Parks, W. Xu, J. Li, M. Gurney, R. L. Myers, C. S. Himes, R. Hiebsch, C. Ruble, J. S. Nye and D. Curtis (2002). "aph-1 and pen-2 are required for Notch pathway signaling, gamma-secretase cleavage of betaAPP, and presenilin protein accumulation." Dev Cell **3**(1): 85-97.

Fu, X. D., A. Mayeda, T. Maniatis and A. R. Krainer (1992). "General splicing factors SF2 and SC35 have equivalent activities in vitro, and both affect alternative 5' and 3' splice site selection." Proc Natl Acad Sci U S A **89**(23): 11224-11228.

Fujita, T., A. Shimada, N. Okada and A. Yamamoto (2006). "Functional characterization of Na+-independent choline transport in primary cultures of neurons from mouse cerebral cortex." Neurosci Lett **393**(2-3): 216-221.

Futami, T., K. Takakusaki and S. T. Kitai (1995). "Glutamatergic and cholinergic inputs from the pedunculopontine tegmental nucleus to dopamine neurons in the substantia nigra pars compacta." Neurosci Res **21**(4): 331-342.

Galante, P. A., N. J. Sakabe, N. Kirschbaum-Slager and S. J. de Souza (2004). "Detection and evaluation of intron retention events in the human transcriptome." RNA **10**(5): 757-765.

Gates, J., Jr., S. M. Ferguson, R. D. Blakely and S. Apparsundaram (2004). "Regulation of choline transporter surface expression and phosphorylation by protein kinase C and protein phosphatase 1/2A." J Pharmacol Exp Ther **310**(2): 536-545.

Gaykema, R. P., P. G. Luiten, C. Nyakas and J. Traber (1990). "Cortical projection patterns of the medial septum-diagonal band complex." J Comp Neurol **293**(1): 103-124.

Ge, S. and J. A. Dani (2005). "Nicotinic acetylcholine receptors at glutamate synapses facilitate long-term depression or potentiation." J Neurosci **25**(26): 6084-6091.

Gerber, D. J., T. D. Sotnikova, R. R. Gainetdinov, S. Y. Huang, M. G. Caron and S. Tonegawa (2001). "Hyperactivity, elevated dopaminergic transmission, and response to amphetamine in M1 muscarinic acetylcholine receptor-deficient mice." Proc Natl Acad Sci U S A **98**(26): 15312-15317.

Ghoneim, M. M. and S. P. Mewaldt (1975). "Effects of diazepam and scopolamine on storage, retrieval and organizational processes in memory." Psychopharmacologia **44**(3): 257-262.

Gibson, G. E. and C. Peterson (1981). "Aging decreases oxidative metabolism and the release and synthesis of acetylcholine." J Neurochem **37**(4): 978-984.

Gibson, G. E., C. Peterson and J. Sansone (1981). "Neurotransmitter and carbohydrate metabolism during aging and mild hypoxia." Neurobiol Aging **2**(3): 165-172.

Gilad, G. M., J. M. Rabey, Y. Tizabi and V. H. Gilad (1987). "Age-dependent loss and compensatory changes of septohippocampal cholinergic neurons in two rat strains differing in longevity and response to stress." Brain Res **436**(2): 311-322.

Gill, S. K., M. Bhattacharya, S. S. Ferguson and R. J. Rylett (2003). "Identification of a novel nuclear localization signal common to 69- and 82-kDa human choline acetyltransferase." J Biol Chem **278**(22): 20217-20224.

Gilmor, M. L., N. R. Nash, A. Roghani, R. H. Edwards, H. Yi, S. M. Hersch and A. I. Levey (1996). "Expression of the putative vesicular acetylcholine transporter in rat brain and localization in cholinergic synaptic vesicles." J Neurosci **16**(7): 2179-2190.

Giniatullin, R., A. Nistri and J. L. Yakel (2005). "Desensitization of nicotinic ACh receptors: shaping cholinergic signaling." Trends Neurosci **28**(7): 371-378.

Goedert, M., R. Jakes, Z. Qi, J. H. Wang and P. Cohen (1995). "Protein phosphatase 2A is the major enzyme in brain that dephosphorylates tau protein phosphorylated by proline-directed protein kinases or cyclic AMP-dependent protein kinase." J Neurochem **65**(6): 2804-2807.

Goedert, M., C. M. Wischik, R. A. Crowther, J. E. Walker and A. Klug (1988). "Cloning and sequencing of the cDNA encoding a core protein of the paired helical filament of Alzheimer disease: identification as the microtubule-associated protein tau." Proc Natl Acad Sci U S A **85**(11): 4051-4055.

Goekoop, R., P. Scheltens, F. Barkhof and S. A. Rombouts (2006). "Cholinergic challenge in Alzheimer patients and mild cognitive impairment differentially affects hippocampal activation--a pharmacological fMRI study." Brain **129**(Pt 1): 141-157.

Goldberg, J. A. and J. N. Reynolds (2011). "Spontaneous firing and evoked pauses in the tonically active cholinergic interneurons of the striatum." Neuroscience **198**: 27-43.

Golde, T. E., S. Estus, M. Usiak, L. H. Younkin and S. G. Younkin (1990). "Expression of beta amyloid protein precursor mRNAs: recognition of a novel alternatively spliced form and quantitation in Alzheimer's disease using PCR." Neuron **4**(2): 253-267.

Gomez-Varela, D. and D. K. Berg (2013). "Lateral mobility of presynaptic alpha7-containing nicotinic receptors and its relevance for glutamate release." J Neurosci **33**(43): 17062-17071.

Gong, Y., L. Chang, K. L. Viola, P. N. Lacor, M. P. Lambert, C. E. Finch, G. A. Krafft and W. L. Klein (2003). "Alzheimer's disease-affected brain: presence of oligomeric A beta ligands (ADDLs) suggests a molecular basis for reversible memory loss." Proc Natl Acad Sci U S A **100**(18): 10417-10422.

Gornstein, E. and T. L. Schwarz (2014). "The paradox of paclitaxel neurotoxicity: Mechanisms and unanswered questions." Neuropharmacology **76 Pt A**: 175-183.

Gould, R. W., D. Dencker, M. Grannan, M. Bubser, X. Zhan, J. Wess, Z. Xiang, C. Locuson, C. W. Lindsley, P. J. Conn and C. K. Jones (2015). "Role for the M1 Muscarinic Acetylcholine Receptor in Top-Down Cognitive Processing Using a Touchscreen Visual Discrimination Task in Mice." ACS Chem Neurosci **6**(10): 1683-1695.

Gould, T. D. and H. K. Manji (2004). "The molecular medicine revolution and psychiatry: bridging the gap between basic neuroscience research and clinical psychiatry." J Clin Psychiatry **65**(5): 598-604.

Goutte, C., M. Tsunozaki, V. A. Hale and J. R. Priess (2002). "APH-1 is a multipass membrane protein essential for the Notch signaling pathway in Caenorhabditis elegans embryos." Proc Natl Acad Sci U S A **99**(2): 775-779.

Govindasamy, L., B. Pedersen, W. Lian, T. Kukar, Y. Gu, S. Jin, M. Agbandje-McKenna, D. Wu and R. McKenna (2004). "Structural insights and functional implications of choline acetyltransferase." J Struct Biol **148**(2): 226-235.

Graham, K. S. and J. R. Hodges (1997). "Differentiating the roles of the hippocampal complex and the neocortex in long-term memory storage: evidence from the study of semantic dementia and Alzheimer's disease." Neuropsychology **11**(1): 77-89.

Granger, A. J., N. Mulder, A. Saunders and B. L. Sabatini (2016). "Cotransmission of acetylcholine and GABA." Neuropharmacology **100**: 40-46.

Granon, S., B. Poucet, C. Thinus-Blanc, J. P. Changeux and C. Vidal (1995). "Nicotinic and muscarinic receptors in the rat prefrontal cortex: differential roles in working memory, response selection and effortful processing." Psychopharmacology (Berl) **119**(2): 139-144.

Gras, C., B. Amilhon, E. M. Lepicard, O. Poirel, J. Vinatier, M. Herbin, S. Dumas, E. T. Tzavara, M. R. Wade, G. G. Nomikos, N. Hanoun, F. Saurini, M. L. Kemel, B. Gasnier, B. Giros and S. El Mestikawy (2008). "The vesicular glutamate transporter VGLUT3 synergizes striatal acetylcholine tone." Nat Neurosci **11**(3): 292-300.

Gray, S. L., M. L. Anderson, S. Dublin, J. T. Hanlon, R. Hubbard, R. Walker, O. Yu, P. K. Crane and E. B. Larson (2015). "Cumulative use of strong anticholinergics and incident dementia: a prospective cohort study." JAMA Intern Med **175**(3): 401-407.

Greenwood, P. M., M. K. Lin, R. Sundararajan, K. J. Fryxell and R. Parasuraman (2009). "Synergistic effects of genetic variation in nicotinic and muscarinic receptors on visual attention but not working memory." Proc Natl Acad Sci U S A **106**(9): 3633-3638.

Grimm, L., E. Holinski-Feder, J. Teodoridis, B. Scheffer, D. Schindelhauer, T. Meitinger and M. Ueffing (1998). "Analysis of the human GDNF gene reveals an inducible promoter, three exons, a triplet repeat within the 3'-UTR and alternative splice products." Hum Mol Genet **7**(12): 1873-1886.

Gritton, H. J., W. M. Howe, C. S. Mallory, V. L. Hetrick, J. D. Berke and M. Sarter (2016). "Cortical cholinergic signaling controls the detection of cues." Proc Natl Acad Sci U S A **113**(8): E1089-1097.

Grottick, A. J. and G. A. Higgins (2000). "Effect of subtype selective nicotinic compounds on attention as assessed by the five-choice serial reaction time task." Behav Brain Res **117**(1-2): 197-208.

Gu, Y., H. Misonou, T. Sato, N. Dohmae, K. Takio and Y. Ihara (2001). "Distinct intramembrane cleavage of the beta-amyloid precursor protein family resembling gamma-secretase-like cleavage of Notch." J Biol Chem **276**(38): 35235-35238.

Gu, Z. and J. L. Yakel (2011). "Timing-dependent septal cholinergic induction of dynamic hippocampal synaptic plasticity." Neuron **71**(1): 155-165.

Guillem, K., B. Bloem, R. B. Poorthuis, M. Loos, A. B. Smit, U. Maskos, S. Spijker and H. D. Mansvelder (2011). "Nicotinic acetylcholine receptor beta2 subunits in the medial prefrontal cortex control attention." Science **333**(6044): 888-891.

Guzman, M. S., X. De Jaeger, S. Raulic, I. A. Souza, A. X. Li, S. Schmid, R. S. Menon, R. R. Gainetdinov, M. G. Caron, R. Bartha, V. F. Prado and M. A. Prado (2011). "Elimination of the vesicular acetylcholine transporter in the striatum reveals regulation of behaviour by cholinergic-glutamatergic co-transmission." PLoS Biol **9**(11): e1001194.

Haapasalo, A. and D. M. Kovacs (2011). "The many substrates of presenilin/gamma-secretase." J Alzheimers Dis **25**(1): 3-28.

Haass, C., A. Y. Hung, M. G. Schlossmacher, D. B. Teplow and D. J. Selkoe (1993). "beta-Amyloid peptide and a 3-kDa fragment are derived by distinct cellular mechanisms." J Biol Chem **268**(5): 3021-3024.

Haass, C. and D. J. Selkoe (2007). "Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide." Nat Rev Mol Cell Biol **8**(2): 101-112.

Haass, C. and H. Steiner (2002). "Alzheimer disease gamma-secretase: a complex story of GxGD-type presenilin proteases." Trends Cell Biol **12**(12): 556-562.

Hardy, J. A. and G. A. Higgins (1992). "Alzheimer's disease: the amyloid cascade hypothesis." Science **256**(5054): 184-185.

Hartig, W., A. Saul, J. Kacza, J. Grosche, S. Goldhammer, D. Michalski and O. Wirths (2014). "Immunolesion-induced loss of cholinergic projection neurones promotes beta-amyloidosis and tau hyperphosphorylation in the hippocampus of triple-transgenic mice." Neuropathol Appl Neurobiol **40**(2): 106-120.

Hasselmo, M. E. (1999). "Neuromodulation: acetylcholine and memory consolidation." Trends Cogn Sci **3**(9): 351-359.

Hasselmo, M. E. (2006). "The role of acetylcholine in learning and memory." Curr Opin Neurobiol **16**(6): 710-715.

Hasselmo, M. E. and J. M. Bower (1993). "Acetylcholine and memory." Trends Neurosci **16**(6): 218-222.

Hasselmo, M. E. and M. Sarter (2011). "Modes and models of forebrain cholinergic neuromodulation of cognition." Neuropsychopharmacology **36**(1): 52-73.

He, G., W. Luo, P. Li, C. Remmers, W. J. Netzer, J. Hendrick, K. Bettayeb, M. Flajolet, F. Gorelick, L. P. Wennogle and P. Greengard (2010). "Gamma-secretase activating protein is a therapeutic target for Alzheimer's disease." Nature **467**(7311): 95-98.

Hebb, C. O. and A. Silver (1961). "Gradient of choline acetylase activity." Nature **189**: 123-125.

Heidbreder, C. A., A. C. Thompson and T. S. Shippenberg (1996). "Role of extracellular dopamine in the initiation and long-term expression of behavioral sensitization to cocaine." J Pharmacol Exp Ther **278**(2): 490-502.

Henny, P. and B. E. Jones (2006). "Innervation of orexin/hypocretin neurons by GABAergic, glutamatergic or cholinergic basal forebrain terminals evidenced by immunostaining for presynaptic vesicular transporter and postsynaptic scaffolding proteins." J Comp Neurol **499**(4): 645-661.

Henny, P. and B. E. Jones (2008). "Projections from basal forebrain to prefrontal cortex comprise cholinergic, GABAergic and glutamatergic inputs to pyramidal cells or interneurons." Eur J Neurosci **27**(3): 654-670.

Hersh, L. B. (1982). "Kinetic studies of the choline acetyltransferase reaction using isotope exchange at equilibrium." J Biol Chem **257**(21): 12820-12825.

Hikida, T., S. Kaneko, T. Isobe, Y. Kitabatake, D. Watanabe, I. Pastan and S. Nakanishi (2001). "Increased sensitivity to cocaine by cholinergic cell ablation in nucleus accumbens." Proc Natl Acad Sci U S A **98**(23): 13351-13354.

Hironaka, N., K. Tanaka, Y. Izaki, K. Hori and M. Nomura (2001). "Memory-related acetylcholine efflux from rat prefrontal cortex and hippocampus: a microdialysis study." Brain Res **901**(1-2): 143-150.

Hodnett, J. L. and H. Busch (1968). "Isolation and characterization of uridylic acid-rich 7 S ribonucleic acid of rat liver nuclei." J Biol Chem **243**(24): 6334-6342.

Hohmann, C. F. and J. Berger-Sweeney (1998). "Cholinergic regulation of cortical development and plasticity. New twists to an old story." Perspect Dev Neurobiol **5**(4): 401-425.

Howe, W. M., J. Ji, V. Parikh, S. Williams, E. Mocaer, C. Trocme-Thibierge and M. Sarter (2010). "Enhancement of attentional performance by selective stimulation of alpha4beta2(*) nAChRs: underlying cholinergic mechanisms." Neuropsychopharmacology **35**(6): 1391-1401.

Hoyle, E., R. F. Genn, C. Fernandes and I. P. Stolerman (2006). "Impaired performance of alpha7 nicotinic receptor knockout mice in the five-choice serial reaction time task." Psychopharmacology (Berl) **189**(2): 211-223.

Hu, X., C. W. Hicks, W. He, P. Wong, W. B. Macklin, B. D. Trapp and R. Yan (2006). "Bace1 modulates myelination in the central and peripheral nervous system." Nat Neurosci **9**(12): 1520-1525.

Huang, C. C. and K. S. Hsu (2010). "Activation of muscarinic acetylcholine receptors induces a nitric oxide-dependent long-term depression in rat medial prefrontal cortex." Cereb Cortex **20**(4): 982-996.

Huh, C. Y., R. Goutagny and S. Williams (2010). "Glutamatergic neurons of the mouse medial septum and diagonal band of Broca synaptically drive hippocampal pyramidal cells: relevance for hippocampal theta rhythm." J Neurosci **30**(47): 15951-15961.

Izaurralde, E., J. Lewis, C. McGuigan, M. Jankowska, E. Darzynkiewicz and I. W. Mattaj (1994). "A nuclear cap binding protein complex involved in pre-mRNA splicing." Cell **78**(4): 657-668.

Jankowsky, J. L., D. J. Fadale, J. Anderson, G. M. Xu, V. Gonzales, N. A. Jenkins, N. G. Copeland, M. K. Lee, L. H. Younkin, S. L. Wagner, S. G. Younkin and D. R. Borchelt (2004). "Mutant presenilins specifically elevate the levels of the 42 residue beta-amyloid peptide in vivo: evidence for augmentation of a 42-specific gamma secretase." Hum Mol Genet **13**(2): 159-170.

Jarrett, J. T., E. P. Berger and P. T. Lansbury, Jr. (1993). "The C-terminus of the beta protein is critical in amyloidogenesis." Ann N Y Acad Sci **695**: 144-148.

Jensen, K. B., B. K. Dredge, G. Stefani, R. Zhong, R. J. Buckanovich, H. J. Okano, Y. Y. Yang and R. B. Darnell (2000). "Nova-1 regulates neuron-specific alternative splicing and is essential for neuronal viability." Neuron **25**(2): 359-371.

Jessen, F., H. Kaduszkiewicz, M. Daerr, H. Bickel, M. Pentzek, S. Riedel-Heller, M. Wagner, S. Weyerer, B. Wiese, H. van den Bussche, K. Broich and W. Maier (2010). "Anticholinergic drug use and risk for dementia: target for dementia prevention." Eur Arch Psychiatry Clin Neurosci **260 Suppl 2**: S111-115.

Jicha, G. A., C. Weaver, E. Lane, C. Vianna, Y. Kress, J. Rockwood and P. Davies (1999). "cAMP-dependent protein kinase phosphorylations on tau in Alzheimer's disease." J Neurosci **19**(17): 7486-7494.

Jo, J., G. H. Son, B. L. Winters, M. J. Kim, D. J. Whitcomb, B. A. Dickinson, Y. B. Lee, K. Futai, M. Amici, M. Sheng, G. L. Collingridge and K. Cho (2010). "Muscarinic receptors induce LTD of NMDAR EPSCs via a mechanism involving hippocalcin, AP2 and PSD-95." Nat Neurosci **13**(10): 1216-1224.

Jurica, M. S., L. J. Licklider, S. R. Gygi, N. Grigorieff and M. J. Moore (2002). "Purification and characterization of native spliceosomes suitable for three-dimensional structural analysis." RNA **8**(4): 426-439.

Kanitz, A., F. Gypas, A. J. Gruber, A. R. Gruber, G. Martin and M. Zavolan (2015). "Comparative assessment of methods for the computational inference of transcript isoform abundance from RNA-seq data." Genome Biol **16**: 150.

Karlin, A. and M. H. Akabas (1995). "Toward a structural basis for the function of nicotinic acetylcholine receptors and their cousins." Neuron **15**(6): 1231-1244.

Karlsson, R. M., K. Tanaka, L. M. Saksida, T. J. Bussey, M. Heilig and A. Holmes (2009). "Assessment of glutamate transporter GLAST (EAAT1)-deficient mice for

phenotypes relevant to the negative and executive/cognitive symptoms of schizophrenia." Neuropsychopharmacology **34**(6): 1578-1589.

Katz, B. and R. Miledi (1965). "The Measurement of Synaptic Delay, and the Time Course of Acetylcholine Release at the Neuromuscular Junction." Proc R Soc Lond B Biol Sci **161**: 483-495.

Katz, B. and S. Thesleff (1957). "On the factors which determine the amplitude of the miniature end-plate potential." J Physiol **137**(2): 267-278.

Khare, P., A. M. Ojeda, A. Chandrasekaran and S. M. Parsons (2010). "Possible important pair of acidic residues in vesicular acetylcholine transporter." Biochemistry **49**(14): 3049-3059.

Kim, A. R., R. J. Rylett and B. H. Shilton (2006). "Substrate binding and catalytic mechanism of human choline acetyltransferase." Biochemistry **45**(49): 14621-14631.

Kim, H. J., N. C. Kim, Y. D. Wang, E. A. Scarborough, J. Moore, Z. Diaz, K. S. MacLea, B. Freibaum, S. Li, A. Molliex, A. P. Kanagaraj, R. Carter, K. B. Boylan, A. M. Wojtas, R. Rademakers, J. L. Pinkus, S. A. Greenberg, J. Q. Trojanowski, B. J. Traynor, B. N. Smith, S. Topp, A. S. Gkazi, J. Miller, C. E. Shaw, M. Kottlors, J. Kirschner, A. Pestronk, Y. R. Li, A. F. Ford, A. D. Gitler, M. Benatar, O. D. King, V. E. Kimonis, E. D. Ross, C. C. Weihl, J. Shorter and J. P. Taylor (2013). "Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS." Nature **495**(7442): 467-473.

King, S. L., B. J. Caldarone and M. R. Picciotto (2004). "Beta2-subunit-containing nicotinic acetylcholine receptors are critical for dopamine-dependent locomotor activation following repeated nicotine administration." Neuropharmacology **47 Suppl 1**: 132-139.

Kirkwood, A., C. Rozas, J. Kirkwood, F. Perez and M. F. Bear (1999). "Modulation of long-term synaptic depression in visual cortex by acetylcholine and norepinephrine." J Neurosci **19**(5): 1599-1609.

Kitabatake, Y., T. Hikida, D. Watanabe, I. Pastan and S. Nakanishi (2003). "Impairment of reward-related learning by cholinergic cell ablation in the striatum." Proc Natl Acad Sci U S A **100**(13): 7965-7970.

Kitamoto, T., W. Wang and P. M. Salvaterra (1998). "Structure and organization of the Drosophila cholinergic locus." J Biol Chem **273**(5): 2706-2713.

Klinkenberg, I. and A. Blokland (2010). "The validity of scopolamine as a pharmacological model for cognitive impairment: a review of animal behavioral studies." Neurosci Biobehav Rev **34**(8): 1307-1350.

Knowlton, B. J., J. A. Mangels and L. R. Squire (1996). "A neostriatal habit learning system in humans." Science **273**(5280): 1399-1402.

Konig, J., K. Zarnack, G. Rot, T. Curk, M. Kayikci, B. Zupan, D. J. Turner, N. M. Luscombe and J. Ule (2010). "iCLIP reveals the function of hnRNP particles in splicing at individual nucleotide resolution." Nat Struct Mol Biol **17**(7): 909-915.

Koshimizu, H., L. M. Leiter and T. Miyakawa (2012). "M4 muscarinic receptor knockout mice display abnormal social behavior and decreased prepulse inhibition." Mol Brain **5**: 10.

Krantz, D. E., C. Waites, V. Oorschot, Y. Liu, R. I. Wilson, P. K. Tan, J. Klumperman and R. H. Edwards (2000). "A phosphorylation site regulates sorting of the vesicular acetylcholine transporter to dense core vesicles." J Cell Biol **149**(2): 379-396.

Kunkel, M. T. and E. G. Peralta (1995). "Identification of domains conferring G protein regulation on inward rectifier potassium channels." Cell **83**(3): 443-449.

Lacor, P. N., M. C. Buniel, L. Chang, S. J. Fernandez, Y. Gong, K. L. Viola, M. P. Lambert, P. T. Velasco, E. H. Bigio, C. E. Finch, G. A. Krafft and W. L. Klein (2004). "Synaptic targeting by Alzheimer's-related amyloid beta oligomers." J Neurosci **24**(45): 10191-10200.

Lacor, P. N., M. C. Buniel, P. W. Furlow, A. S. Clemente, P. T. Velasco, M. Wood, K. L. Viola and W. L. Klein (2007). "Abeta oligomer-induced aberrations in synapse composition, shape, and density provide a molecular basis for loss of connectivity in Alzheimer's disease." J Neurosci **27**(4): 796-807.

Lambe, E. K., M. R. Picciotto and G. K. Aghajanian (2003). "Nicotine induces glutamate release from thalamocortical terminals in prefrontal cortex." Neuropsychopharmacology **28**(2): 216-225.

Lambert, M. P., A. K. Barlow, B. A. Chromy, C. Edwards, R. Freed, M. Liosatos, T. E. Morgan, I. Rozovsky, B. Trommer, K. L. Viola, P. Wals, C. Zhang, C. E. Finch, G. A.

Krafft and W. L. Klein (1998). "Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins." Proc Natl Acad Sci U S A **95**(11): 6448-6453.

Lauren, J., D. A. Gimbel, H. B. Nygaard, J. W. Gilbert and S. M. Strittmatter (2009). "Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers." Nature **457**(7233): 1128-1132.

Le Novere, N. and J. P. Changeux (1995). "Molecular evolution of the nicotinic acetylcholine receptor: an example of multigene family in excitable cells." J Mol Evol **40**(2): 155-172.

Leal, G., P. M. Afonso and C. B. Duarte (2014). "Neuronal activity induces synaptic delivery of hnRNP A2/B1 by a BDNF-dependent mechanism in cultured hippocampal neurons." PLoS One **9**(10): e108175.

Lee, G., N. Cowan and M. Kirschner (1988). "The primary structure and heterogeneity of tau protein from mouse brain." Science **239**(4837): 285-288.

Leem, J. Y., C. A. Saura, C. Pietrzik, J. Christianson, C. Wanamaker, L. T. King, M. L. Veselits, T. Tomita, L. Gasparini, T. Iwatsubo, H. Xu, W. N. Green, E. H. Koo and G. Thinakaran (2002). "A role for presenilin 1 in regulating the delivery of amyloid precursor protein to the cell surface." Neurobiol Dis **11**(1): 64-82.

Lemmens, R., M. J. Moore, A. Al-Chalabi, R. H. Brown, Jr. and W. Robberecht (2010). "RNA metabolism and the pathogenesis of motor neuron diseases." Trends Neurosci **33**(5): 249-258.

Leung, L. S., B. Shen, N. Rajakumar and J. Ma (2003). "Cholinergic activity enhances hippocampal long-term potentiation in CA1 during walking in rats." J Neurosci **23**(28): 9297-9304.

Levey, A. I. (1993). "Immunological localization of m1-m5 muscarinic acetylcholine receptors in peripheral tissues and brain." Life Sci **52**(5-6): 441-448.

Levey, A. I., S. M. Edmunds, V. Koliatsos, R. G. Wiley and C. J. Heilman (1995). "Expression of m1-m4 muscarinic acetylcholine receptor proteins in rat hippocampus and regulation by cholinergic innervation." J Neurosci **15**(5 Pt 2): 4077-4092.

Lewis, P. R. and C. C. Shute (1967). "The cholinergic limbic system: projections to hippocampal formation, medial cortex, nuclei of the ascending cholinergic reticular system, and the subfornical organ and supra-optic crest." Brain **90**(3): 521-540.

Li, Q., J. A. Lee and D. L. Black (2007). "Neuronal regulation of alternative pre-mRNA splicing." Nat Rev Neurosci **8**(11): 819-831.

Lilja, A. M., O. Porras, E. Storelli, A. Nordberg and A. Marutle (2011). "Functional interactions of fibrillar and oligomeric amyloid-beta with alpha7 nicotinic receptors in Alzheimer's disease." J Alzheimers Dis **23**(2): 335-347.

Lima Rde, F., V. F. Prado, M. A. Prado and C. Kushmerick (2010). "Quantal release of acetylcholine in mice with reduced levels of the vesicular acetylcholine transporter." J Neurochem **113**(4): 943-951.

Lin, H., R. Bhatia and R. Lal (2001). "Amyloid beta protein forms ion channels: implications for Alzheimer's disease pathophysiology." FASEB J **15**(13): 2433-2444.

Linder, T. M., P. Pennefather and D. M. Quastel (1984). "The time course of miniature endplate currents and its modification by receptor blockade and ethanol." J Gen Physiol **83**(3): 435-468.

Lindsay, J., D. Laurin, R. Verreault, R. Hebert, B. Helliwell, G. B. Hill and I. McDowell (2002). "Risk factors for Alzheimer's disease: a prospective analysis from the Canadian Study of Health and Aging." Am J Epidemiol **156**(5): 445-453.

Ling, S. C., M. Polymenidou and D. W. Cleveland (2013). "Converging mechanisms in ALS and FTD: disrupted RNA and protein homeostasis." Neuron **79**(3): 416-438.

Liu, F., I. Grundke-Iqbal, K. Iqbal and C. X. Gong (2005). "Contributions of protein phosphatases PP1, PP2A, PP2B and PP5 to the regulation of tau phosphorylation." Eur J Neurosci **22**(8): 1942-1950.

Liu, Y. and R. H. Edwards (1997). "The role of vesicular transport proteins in synaptic transmission and neural degeneration." Annu Rev Neurosci **20**: 125-156.

Liu, Y., D. Peter, A. Roghani, S. Schuldiner, G. G. Prive, D. Eisenberg, N. Brecha and R. H. Edwards (1992). "A cDNA that suppresses MPP+ toxicity encodes a vesicular amine transporter." Cell **70**(4): 539-551.

Liu, Z., Y. Otsu, C. Vasuta, H. Nawa and T. H. Murphy (2007). "Action-potential-independent GABAergic tone mediated by nicotinic stimulation of immature striatal miniature synaptic transmission." J Neurophysiol **98**(2): 581-593.

Loewi, O. (1921). "Über humorale übertragbarkeit der herznervenwirkung." Pflüger's Archiv für die gesamte Physiologie des Menschen und der Tiere **189**(1): 239-242.

Luo, R., M. J. Janssen, J. G. Partridge and S. Vicini (2013). "Direct and GABA-mediated indirect effects of nicotinic ACh receptor agonists on striatal neurones." J Physiol **591**(1): 203-217.

Luo, Y., B. Bolon, S. Kahn, B. D. Bennett, S. Babu-Khan, P. Denis, W. Fan, H. Kha, J. Zhang, Y. Gong, L. Martin, J. C. Louis, Q. Yan, W. G. Richards, M. Citron and R. Vassar (2001). "Mice deficient in BACE1, the Alzheimer's beta-secretase, have normal phenotype and abolished beta-amyloid generation." Nat Neurosci **4**(3): 231-232.

Ma, Q. L., F. Yang, E. R. Rosario, O. J. Ubeda, W. Beech, D. J. Gant, P. P. Chen, B. Hudspeth, C. Chen, Y. Zhao, H. V. Vinters, S. A. Frautschy and G. M. Cole (2009). "Beta-amyloid oligomers induce phosphorylation of tau and inactivation of insulin receptor substrate via c-Jun N-terminal kinase signaling: suppression by omega-3 fatty acids and curcumin." J Neurosci **29**(28): 9078-9089.

Maccioni, R. B., C. I. Rivas and J. C. Vera (1988). "Differential interaction of synthetic peptides from the carboxyl-terminal regulatory domain of tubulin with microtubule-associated proteins." EMBO J **7**(7): 1957-1963.

Mallet, J., L. Houhou, F. Pajak, Y. Oda, R. Cervini, S. Bejanin and S. Berrard (1998). "The cholinergic locus: ChAT and VAChT genes." J Physiol Paris **92**(2): 145-147.

Manczak, M., T. S. Anekonda, E. Henson, B. S. Park, J. Quinn and P. H. Reddy (2006). "Mitochondria are a direct site of A beta accumulation in Alzheimer's disease neurons: implications for free radical generation and oxidative damage in disease progression." Hum Mol Genet **15**(9): 1437-1449.

Marchi, M., F. Risso, C. Viola, P. Cavazzani and M. Raiteri (2002). "Direct evidence that release-stimulating alpha7* nicotinic cholinergic receptors are localized on human and rat brain glutamatergic axon terminals." J Neurochem **80**(6): 1071-1078.

Marnay, A. and D. Nachmansohn (1937). "Cholinesterase in voluntary frog's muscle." J Physiol **89**(4): 359-367.

Martinez-Contreras, R., P. Cloutier, L. Shkreta, J. F. Fisette, T. Revil and B. Chabot (2007). "hnRNP proteins and splicing control." Adv Exp Med Biol **623**: 123-147.

Martinez-Gonzalez, C., H. L. Wang, B. R. Micklem, J. P. Bolam and J. Mena-Segovia (2012). "Subpopulations of cholinergic, GABAergic and glutamatergic neurons in the pedunculopontine nucleus contain calcium-binding proteins and are heterogeneously distributed." Eur J Neurosci **35**(5): 723-734.

Martyn, A. C., X. De Jaeger, A. C. Magalhaes, R. Kesarwani, D. F. Goncalves, S. Raulic, M. S. Guzman, M. F. Jackson, I. Izquierdo, J. F. Macdonald, M. A. Prado and V. F. Prado (2012). "Elimination of the vesicular acetylcholine transporter in the forebrain causes hyperactivity and deficits in spatial memory and long-term potentiation." Proc Natl Acad Sci U S A **109**(43): 17651-17656.

Maskos, U. (2008). "The cholinergic mesopontine tegmentum is a relatively neglected nicotinic master modulator of the dopaminergic system: relevance to drugs of abuse and pathology." Br J Pharmacol **153 Suppl 1**: S438-445.

Matsui, M., D. Motomura, T. Fujikawa, J. Jiang, S. Takahashi, T. Manabe and M. M. Taketo (2002). "Mice lacking M2 and M3 muscarinic acetylcholine receptors are devoid of cholinergic smooth muscle contractions but still viable." J Neurosci **22**(24): 10627-10632.

Matsuo, A., J. P. Bellier, M. Nishimura, O. Yasuhara, N. Saito and H. Kimura (2011). "Nuclear choline acetyltransferase activates transcription of a high-affinity choline transporter." J Biol Chem **286**(7): 5836-5845.

Mayeda, A. and A. R. Krainer (1992). "Regulation of alternative pre-mRNA splicing by hnRNP A1 and splicing factor SF2." Cell **68**(2): 365-375.

McClure-Begley, T. D., N. M. King, A. C. Collins, J. A. Stitzel, J. M. Wehner and C. M. Butt (2009). "Acetylcholine-stimulated [3H]GABA release from mouse brain synaptosomes is modulated by alpha4beta2 and alpha4alpha5beta2 nicotinic receptor subtypes." Mol Pharmacol **75**(4): 918-926.

McCool, M. F., S. Patel, R. Talati and M. E. Ragozzino (2008). "Differential involvement of M1-type and M4-type muscarinic cholinergic receptors in the dorsomedial striatum in task switching." Neurobiol Learn Mem **89**(2): 114-124.

McEwen, B. S. (1999). "Stress and hippocampal plasticity." Annu Rev Neurosci **22**: 105-122.

McGaughy, J., M. W. Decker and M. Sarter (1999). "Enhancement of sustained attention performance by the nicotinic acetylcholine receptor agonist ABT-418 in intact but not basal forebrain-lesioned rats." Psychopharmacology (Berl) **144**(2): 175-182.

McGaughy, J., T. Kaiser and M. Sarter (1996). "Behavioral vigilance following infusions of 192 IgG-saporin into the basal forebrain: selectivity of the behavioral impairment and relation to cortical AChE-positive fiber density." Behav Neurosci **110**(2): 247-265.

McGaughy, J. and M. Sarter (1998). "Sustained attention performance in rats with intracortical infusions of 192 IgG-saporin-induced cortical cholinergic deafferentation: effects of physostigmine and FG 7142." Behav Neurosci **112**(6): 1519-1525.

McGlincy, N. J. and C. W. Smith (2008). "Alternative splicing resulting in nonsense-mediated mRNA decay: what is the meaning of nonsense?" Trends Biochem Sci **33**(8): 385-393.

Mesulam, M. M., E. J. Mufson, A. I. Levey and B. H. Wainer (1983). "Cholinergic innervation of cortex by the basal forebrain: cytochemistry and cortical connections of the septal area, diagonal band nuclei, nucleus basalis (substantia innominata), and hypothalamus in the rhesus monkey." J Comp Neurol **214**(2): 170-197.

Metherate, R., C. L. Cox and J. H. Ashe (1992). "Cellular bases of neocortical activation: modulation of neural oscillations by the nucleus basalis and endogenous acetylcholine." J Neurosci **12**(12): 4701-4711.

Meyer, E. M., F. T. Crews, D. H. Otero and K. Larsen (1986). "Aging decreases the sensitivity of rat cortical synaptosomes to calcium ionophore-induced acetylcholine release." J Neurochem **47**(4): 1244-1246.

Migeon, J. C. and N. M. Nathanson (1994). "Differential regulation of cAMP-mediated gene transcription by m1 and m4 muscarinic acetylcholine receptors. Preferential coupling of m4 receptors to Gi alpha-2." J Biol Chem **269**(13): 9767-9773.

Mileusnic, R., C. L. Lancashire, A. N. Johnston and S. P. Rose (2000). "APP is required during an early phase of memory formation." Eur J Neurosci **12**(12): 4487-4495.

Millecamps, S. and J. P. Julien (2013). "Axonal transport deficits and neurodegenerative diseases." Nat Rev Neurosci **14**(3): 161-176.

Miller, J. A., M. C. Oldham and D. H. Geschwind (2008). "A systems level analysis of transcriptional changes in Alzheimer's disease and normal aging." J Neurosci **28**(6): 1410-1420.

Minoo, P., T. E. Martin and R. M. Riehl (1991). "Nucleic acid binding characteristics of group A/B hnRNP proteins." Biochem Biophys Res Commun **176**(2): 747-755.

Misawa, H., K. Ishii and T. Deguchi (1992). "Gene expression of mouse choline acetyltransferase. Alternative splicing and identification of a highly active promoter region." J Biol Chem **267**(28): 20392-20399.

Misawa, H., K. Nakata, J. Matsuura, M. Nagao, T. Okuda and T. Haga (2001). "Distribution of the high-affinity choline transporter in the central nervous system of the rat." Neuroscience **105**(1): 87-98.

Misgeld, T., R. W. Burgess, R. M. Lewis, J. M. Cunningham, J. W. Lichtman and J. R. Sanes (2002). "Roles of neurotransmitter in synapse formation: development of neuromuscular junctions lacking choline acetyltransferase." Neuron **36**(4): 635-648.

Miyamoto, M., M. Shintani, A. Nagaoka and Y. Nagawa (1985). "Lesioning of the rat basal forebrain leads to memory impairments in passive and active avoidance tasks." Brain Res **328**(1): 97-104.

Moore, H., S. Stuckman, M. Sarter and J. P. Bruno (1996). "Potassium, but not atropine-stimulated cortical acetylcholine efflux, is reduced in aged rats." Neurobiol Aging **17**(4): 565-571.

Morishita, H., J. M. Miwa, N. Heintz and T. K. Hensch (2010). "Lynx1, a cholinergic brake, limits plasticity in adult visual cortex." Science **330**(6008): 1238-1240.

Morris, J. C. (2002). "Challenging assumptions about Alzheimer's disease: mild cognitive impairment and the cholinergic hypothesis." Ann Neurol **51**(2): 143-144.

Mortazavi, A., B. A. Williams, K. McCue, L. Schaeffer and B. Wold (2008). "Mapping and quantifying mammalian transcriptomes by RNA-Seq." Nat Methods **5**(7): 621-628.

Motelow, J. E., W. Li, Q. Zhan, A. M. Mishra, R. N. Sachdev, G. Liu, A. Gummadavelli, Z. Zayyad, H. S. Lee, V. Chu, J. P. Andrews, D. J. Englot, P. Herman, B. G. Sanganahalli, F. Hyder and H. Blumenfeld (2015). "Decreased subcortical cholinergic arousal in focal seizures." Neuron **85**(3): 561-572.

Mount, S. M., I. Pettersson, M. Hinterberger, A. Karmas and J. A. Steitz (1983). "The U1 small nuclear RNA-protein complex selectively binds a 5' splice site in vitro." Cell **33**(2): 509-518.

Mufson, E. J., S. Y. Ma, J. Dills, E. J. Cochran, S. Leurgans, J. Wuu, D. A. Bennett, S. Jaffar, M. L. Gilmor, A. I. Levey and J. H. Kordower (2002). "Loss of basal forebrain P75(NTR) immunoreactivity in subjects with mild cognitive impairment and Alzheimer's disease." J Comp Neurol **443**(2): 136-153.

Muir, J. L., S. B. Dunnett, T. W. Robbins and B. J. Everitt (1992). "Attentional functions of the forebrain cholinergic systems: effects of intraventricular hemicholinium, physostigmine, basal forebrain lesions and intracortical grafts on a multiple-choice serial reaction time task." Exp Brain Res **89**(3): 611-622.

Muller, U. C. and H. Zheng (2012). "Physiological functions of APP family proteins." Cold Spring Harb Perspect Med **2**(2): a006288.

Myer, V. E. and J. A. Steitz (1995). "Isolation and characterization of a novel, low abundance hnRNP protein: A0." RNA **1**(2): 171-182.

Nachmansohn, D. and M. Berman (1946). "Studies on choline acetylase; on the preparation of the coenzyme and its effect on the enzyme." J Biol Chem **165**(2): 551-563.

Nakanishi, T., T. Haruta, Y. Shirasaka and I. Tamai (2011). "Organic cation transporter-mediated renal secretion of ipratropium and tiotropium in rats and humans." Drug Metab Dispos **39**(1): 117-122.

Nakata, T., T. Matsui, K. Kobayashi, Y. Kobayashi and N. Anzai (2013). "Organic cation transporter 2 (SLC22A2), a low-affinity and high-capacity choline transporter, is preferentially enriched on synaptic vesicles in cholinergic neurons." Neuroscience **252**: 212-221.

Nestor, P. J., P. Scheltens and J. R. Hodges (2004). "Advances in the early detection of Alzheimer's disease." Nat Med **10 Suppl**: S34-41.

Netzer, W. J., F. Dou, D. Cai, D. Veach, S. Jean, Y. Li, W. G. Bornmann, B. Clarkson, H. Xu and P. Greengard (2003). "Gleevec inhibits beta-amyloid production but not Notch cleavage." Proc Natl Acad Sci U S A **100**(21): 12444-12449.

Nguyen, M. L., G. D. Cox and S. M. Parsons (1998). "Kinetic parameters for the vesicular acetylcholine transporter: two protons are exchanged for one acetylcholine." Biochemistry **37**(38): 13400-13410.

Nguyen, T. V., L. Shen, L. Vander Griend, L. N. Quach, N. P. Belichenko, N. Saw, T. Yang, M. Shamloo, T. Wyss-Coray, S. M. Massa and F. M. Longo (2014). "Small molecule p75NTR ligands reduce pathological phosphorylation and misfolding of tau, inflammatory changes, cholinergic degeneration, and cognitive deficits in AbetaPP(L/S) transgenic mice." J Alzheimers Dis **42**(2): 459-483.

Niblock, M. and J. M. Gallo (2012). "Tau alternative splicing in familial and sporadic tauopathies." Biochem Soc Trans **40**(4): 677-680.

Nilsson, L., A. Nordberg, J. Hardy, P. Wester and B. Winblad (1986). "Physostigmine restores 3H-acetylcholine efflux from Alzheimer brain slices to normal level." J Neural Transm **67**(3-4): 275-285.

Nirenberg, M. J., J. Chan, Y. Liu, R. H. Edwards and V. M. Pickel (1996). "Ultrastructural localization of the vesicular monoamine transporter-2 in midbrain dopaminergic neurons: potential sites for somatodendritic storage and release of dopamine." J Neurosci **16**(13): 4135-4145.

Nirenberg, M. J., Y. Liu, D. Peter, R. H. Edwards and V. M. Pickel (1995). "The vesicular monoamine transporter 2 is present in small synaptic vesicles and preferentially localizes to large dense core vesicles in rat solitary tract nuclei." Proc Natl Acad Sci U S A **92**(19): 8773-8777.

Offermanns, S., T. Wieland, D. Homann, J. Sandmann, E. Bombien, K. Spicher, G. Schultz and K. H. Jakobs (1994). "Transfected muscarinic acetylcholine receptors selectively couple to Gi-type G proteins and Gq/11." Mol Pharmacol **45**(5): 890-898.

Ojeda, A. M., N. G. Kolmakova and S. M. Parsons (2004). "Acetylcholine binding site in the vesicular acetylcholine transporter." Biochemistry **43**(35): 11163-11174.

Okaty, B. W., K. Sugino and S. B. Nelson (2011). "Cell type-specific transcriptomics in the brain." J Neurosci **31**(19): 6939-6943.

Okuda, T., T. Haga, Y. Kanai, H. Endou, T. Ishihara and I. Katsura (2000). "Identification and characterization of the high-affinity choline transporter." Nat Neurosci **3**(2): 120-125.

Packard, M. G., R. Hirsh and N. M. White (1989). "Differential effects of fornix and caudate nucleus lesions on two radial maze tasks: evidence for multiple memory systems." J Neurosci **9**(5): 1465-1472.

Packard, M. G. and L. A. Teather (1997). "Double dissociation of hippocampal and dorsal-striatal memory systems by posttraining intracerebral injections of 2-amino-5-phosphonopentanoic acid." Behav Neurosci **111**(3): 543-551.

Padgett, R. A., P. J. Grabowski, M. M. Konarska, S. Seiler and P. A. Sharp (1986). "Splicing of messenger RNA precursors." Annu Rev Biochem **55**: 1119-1150.

Padurariu, M., A. Ciobica, I. Mavroudis, D. Fotiou and S. Baloyannis (2012). "Hippocampal neuronal loss in the CA1 and CA3 areas of Alzheimer's disease patients." Psychiatr Danub **24**(2): 152-158.

Pahapill, P. A. and A. M. Lozano (2000). "The pedunculopontine nucleus and Parkinson's disease." Brain **123 (Pt 9)**: 1767-1783.

Parent, A., D. Pare, Y. Smith and M. Steriade (1988). "Basal forebrain cholinergic and noncholinergic projections to the thalamus and brainstem in cats and monkeys." J Comp Neurol **277**(2): 281-301.

Parent, M. J., M. A. Bedard, A. Aliaga, L. Minuzzi, N. Mechawar, J. P. Soucy, E. Schirrmacher, A. Kostikov, S. G. Gauthier and P. Rosa-Neto (2013). "Cholinergic Depletion in Alzheimer's Disease Shown by [(18) F]FEOBV Autoradiography." Int J Mol Imaging **2013**: 205045.

Parikh, V., R. Kozak, V. Martinez and M. Sarter (2007). "Prefrontal acetylcholine release controls cue detection on multiple timescales." Neuron **56**(1): 141-154.

Parikh, V., K. Man, M. W. Decker and M. Sarter (2008). "Glutamatergic contributions to nicotinic acetylcholine receptor agonist-evoked cholinergic transients in the prefrontal cortex." J Neurosci **28**(14): 3769-3780.

Park, J. J., M. C. Gondre-Lewis, L. E. Eiden and Y. P. Loh (2011). "A distinct trans-Golgi network subcompartment for sorting of synaptic and granule proteins in neurons and neuroendocrine cells." J Cell Sci **124**(Pt 5): 735-744.

Parsons, S. M. (2000). "Transport mechanisms in acetylcholine and monoamine storage." FASEB J **14**(15): 2423-2434.

Passetti, F., J. W. Dalley, M. T. O'Connell, B. J. Everitt and T. W. Robbins (2000). "Increased acetylcholine release in the rat medial prefrontal cortex during performance of a visual attentional task." Eur J Neurosci **12**(8): 3051-3058.

Paylor, R., M. Nguyen, J. N. Crawley, J. Patrick, A. Beaudet and A. Orr-Urtreger (1998). "Alpha7 nicotinic receptor subunits are not necessary for hippocampal-dependent learning or sensorimotor gating: a behavioral characterization of Acra7-deficient mice." Learn Mem **5**(4-5): 302-316.

Pearse, B. M., C. J. Smith and D. J. Owen (2000). "Clathrin coat construction in endocytosis." Curr Opin Struct Biol **10**(2): 220-228.

Perez-Garci, E., J. Bargas and E. Galarraga (2003). "The role of Ca2+ channels in the repetitive firing of striatal projection neurons." Neuroreport **14**(9): 1253-1256.

Perry, E. K., P. H. Gibson, G. Blessed, R. H. Perry and B. E. Tomlinson (1977). "Neurotransmitter enzyme abnormalities in senile dementia. Choline acetyltransferase and glutamic acid decarboxylase activities in necropsy brain tissue." J Neurol Sci **34**(2): 247-265.

Perry, E. K., L. Kilford, A. J. Lees, D. J. Burn and R. H. Perry (2003). "Increased Alzheimer pathology in Parkinson's disease related to antimuscarinic drugs." Ann Neurol **54**(2): 235-238.

Picciotto, M. R., M. J. Higley and Y. S. Mineur (2012). "Acetylcholine as a neuromodulator: cholinergic signaling shapes nervous system function and behavior." Neuron **76**(1): 116-129.

Picciotto, M. R., M. Zoli, R. Rimondini, C. Lena, L. M. Marubio, E. M. Pich, K. Fuxe and J. P. Changeux (1998). "Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine." Nature **391**(6663): 173-177.

Pichat, P., O. E. Bergis, J. P. Terranova, A. Urani, C. Duarte, V. Santucci, C. Gueudet, C. Voltz, R. Steinberg, J. Stemmelin, F. Oury-Donat, P. Avenet, G. Griebel and B. Scatton (2007). "SSR180711, a novel selective alpha7 nicotinic receptor partial agonist: (II) efficacy in experimental models predictive of activity against cognitive symptoms of schizophrenia." Neuropsychopharmacology **32**(1): 17-34.

Pidoplichko, V. I., M. DeBiasi, J. T. Williams and J. A. Dani (1997). "Nicotine activates and desensitizes midbrain dopamine neurons." Nature **390**(6658): 401-404.

Pinol-Roma, S., M. S. Swanson, J. G. Gall and G. Dreyfuss (1989). "A novel heterogeneous nuclear RNP protein with a unique distribution on nascent transcripts." J Cell Biol **109**(6 Pt 1): 2575-2587.

Pisano, C., G. Pratesi, D. Laccabue, F. Zunino, P. Lo Giudice, A. Bellucci, L. Pacifici, B. Camerini, L. Vesci, M. Castorina, S. Cicuzza, G. Tredici, P. Marmiroli, G. Nicolini, S. Galbiati, M. Calvani, P. Carminati and G. Cavaletti (2003). "Paclitaxel and Cisplatin-induced neurotoxicity: a protective role of acetyl-L-carnitine." Clin Cancer Res **9**(15): 5756-5767.

Poorthuis, R. B., B. Bloem, B. Schak, J. Wester, C. P. de Kock and H. D. Mansvelder (2013). "Layer-specific modulation of the prefrontal cortex by nicotinic acetylcholine receptors." Cereb Cortex **23**(1): 148-161.

Portelius, E., A. J. Tran, U. Andreasson, R. Persson, G. Brinkmalm, H. Zetterberg, K. Blennow and A. Westman-Brinkmalm (2007). "Characterization of amyloid beta peptides in cerebrospinal fluid by an automated immunoprecipitation procedure followed by mass spectrometry." J Proteome Res **6**(11): 4433-4439.

Potter, A., J. Corwin, J. Lang, M. Piasecki, R. Lenox and P. A. Newhouse (1999). "Acute effects of the selective cholinergic channel activator (nicotinic agonist) ABT-418 in Alzheimer's disease." Psychopharmacology (Berl) **142**(4): 334-342.

Power, A. E., L. J. Thal and J. L. McGaugh (2002). "Lesions of the nucleus basalis magnocellularis induced by 192 IgG-saporin block memory enhancement with posttraining norepinephrine in the basolateral amygdala." Proc Natl Acad Sci U S A **99**(4): 2315-2319.

Prado, M. A., M. V. Gomez and B. Collier (1992). "Mobilization of the readily releasable pool of acetylcholine from a sympathetic ganglion by tityustoxin in the presence of vesamicol." J Neurochem **59**(2): 544-552.

Prado, M. A., R. A. Reis, V. F. Prado, M. C. de Mello, M. V. Gomez and F. G. de Mello (2002). "Regulation of acetylcholine synthesis and storage." Neurochem Int **41**(5): 291-299.

Prado, V. F., C. Martins-Silva, B. M. de Castro, R. F. Lima, D. M. Barros, E. Amaral, A. J. Ramsey, T. D. Sotnikova, M. R. Ramirez, H. G. Kim, J. I. Rossato, J. Koenen, H. Quan, V. R. Cota, M. F. Moraes, M. V. Gomez, C. Guatimosim, W. C. Wetsel, C.

Kushmerick, G. S. Pereira, R. R. Gainetdinov, I. Izquierdo, M. G. Caron and M. A. Prado (2006). "Mice deficient for the vesicular acetylcholine transporter are myasthenic and have deficits in object and social recognition." Neuron **51**(5): 601-612.

Prado, V. F. and M. A. Prado (2002). "Signals involved in targeting membrane proteins to synaptic vesicles." Cell Mol Neurobiol **22**(5-6): 565-577.

Prado, V. F., A. Roy, B. Kolisnyk, R. Gros and M. A. Prado (2013). "Regulation of cholinergic activity by the vesicular acetylcholine transporter." Biochem J **450**(2): 265-274.

Priller, C., T. Bauer, G. Mitteregger, B. Krebs, H. A. Kretzschmar and J. Herms (2006). "Synapse formation and function is modulated by the amyloid precursor protein." J Neurosci **26**(27): 7212-7221.

Proudfoot, N. J., A. Furger and M. J. Dye (2002). "Integrating mRNA processing with transcription." Cell **108**(4): 501-512.

Puig, K. L. and C. K. Combs (2013). "Expression and function of APP and its metabolites outside the central nervous system." Exp Gerontol **48**(7): 608-611.

Qin, K., C. Dong, G. Wu and N. A. Lambert (2011). "Inactive-state preassembly of G(q)-coupled receptors and G(q) heterotrimers." Nat Chem Biol **7**(10): 740-747.

Rabin, S. J., J. M. Kim, M. Baughn, R. T. Libby, Y. J. Kim, Y. Fan, A. La Spada, B. Stone and J. Ravits (2010). "Sporadic ALS has compartment-specific aberrant exon splicing and altered cell-matrix adhesion biology." Hum Mol Genet **19**(2): 313-328.

Rada, P., G. P. Mark, E. Pothos and B. G. Hoebel (1991). "Systemic morphine simultaneously decreases extracellular acetylcholine and increases dopamine in the nucleus accumbens of freely moving rats." Neuropharmacology **30**(10): 1133-1136.

Ragozzino, M. E., S. Detrick and R. P. Kesner (1999). "Involvement of the prelimbic-infralimbic areas of the rodent prefrontal cortex in behavioral flexibility for place and response learning." J Neurosci **19**(11): 4585-4594.

Ragozzino, M. E., J. Jih and A. Tzavos (2002). "Involvement of the dorsomedial striatum in behavioral flexibility: role of muscarinic cholinergic receptors." Brain Res **953**(1-2): 205-214.

Ragozzino, M. E., J. Kim, D. Hassert, N. Minniti and C. Kiang (2003). "The contribution of the rat prelimbic-infralimbic areas to different forms of task switching." Behav Neurosci **117**(5): 1054-1065.

Raj, B. and B. J. Blencowe (2015). "Alternative Splicing in the Mammalian Nervous System: Recent Insights into Mechanisms and Functional Roles." Neuron **87**(1): 14-27.

Rebane, A., A. Aab and J. A. Steitz (2004). "Transportins 1 and 2 are redundant nuclear import factors for hnRNP A1 and HuR." RNA **10**(4): 590-599.

Ren, K., V. Puig, R. L. Papke, Y. Itoh, J. A. Hughes and E. M. Meyer (2005). "Multiple calcium channels and kinases mediate alpha7 nicotinic receptor neuroprotection in PC12 cells." J Neurochem **94**(4): 926-933.

Rezvani, A. H., E. Kholdebarin, F. H. Brucato, P. M. Callahan, D. A. Lowe and E. D. Levin (2009). "Effect of R3487/MEM3454, a novel nicotinic alpha7 receptor partial agonist and 5-HT3 antagonist on sustained attention in rats." Prog Neuropsychopharmacol Biol Psychiatry **33**(2): 269-275.

Ribeiro, F. M., J. Alves-Silva, W. Volknandt, C. Martins-Silva, H. Mahmud, A. Wilhelm, M. V. Gomez, R. J. Rylett, S. S. Ferguson, V. F. Prado and M. A. Prado (2003). "The hemicholinium-3 sensitive high affinity choline transporter is internalized by clathrin-mediated endocytosis and is present in endosomes and synaptic vesicles." J Neurochem **87**(1): 136-146.

Ribeiro, F. M., S. A. Black, S. P. Cregan, V. F. Prado, M. A. Prado, R. J. Rylett and S. S. Ferguson (2005). "Constitutive high-affinity choline transporter endocytosis is determined by a carboxyl-terminal tail dileucine motif." J Neurochem **94**(1): 86-96.

Risacher, S. L., B. C. McDonald, E. F. Tallman, J. D. West, M. R. Farlow, F. W. Unverzagt, S. Gao, M. Boustani, P. K. Crane, R. C. Petersen, C. R. Jack, Jr., W. J. Jagust, P. S. Aisen, M. W. Weiner and A. J. Saykin (2016). "Association Between Anticholinergic Medication Use and Cognition, Brain Metabolism, and Brain Atrophy in Cognitively Normal Older Adults." JAMA Neurol.

Robbins, T. W., B. J. Everitt, H. M. Marston, J. Wilkinson, G. H. Jones and K. J. Page (1989). "Comparative effects of ibotenic acid- and quisqualic acid-induced lesions of the substantia innominata on attentional function in the rat: further implications for the role

of the cholinergic neurons of the nucleus basalis in cognitive processes." Behav Brain Res **35**(3): 221-240.

Rogers, J. T., A. I. Bush, H. H. Cho, D. H. Smith, A. M. Thomson, A. L. Friedlich, D. K. Lahiri, P. J. Leedman, X. Huang and C. M. Cahill (2008). "Iron and the translation of the amyloid precursor protein (APP) and ferritin mRNAs: riboregulation against neural oxidative damage in Alzheimer's disease." Biochem Soc Trans **36**(Pt 6): 1282-1287.

Roghani, A., J. Feldman, S. A. Kohan, A. Shirzadi, C. B. Gundersen, N. Brecha and R. H. Edwards (1994). "Molecular cloning of a putative vesicular transporter for acetylcholine." Proc Natl Acad Sci U S A **91**(22): 10620-10624.

Rusted, J. M. and D. M. Warburton (1992). "Facilitation of memory by post-trial administration of nicotine: evidence for an attentional explanation." Psychopharmacology (Berl) **108**(4): 452-455.

Rylett, R. J., M. J. Ball and E. H. Colhoun (1983). "Evidence for high affinity choline transport in synaptosomes prepared from hippocampus and neocortex of patients with Alzheimer's disease." Brain Res **289**(1-2): 169-175.

Rylett, R. J. and B. M. Schmidt (1993). "Regulation of the synthesis of acetylcholine." Prog Brain Res **98**: 161-166.

Sakae, D. Y., F. Marti, S. Lecca, F. Vorspan, E. Martin-Garcia, L. J. Morel, A. Henrion, J. Gutierrez-Cuesta, A. Besnard, N. Heck, E. Herzog, S. Bolte, V. F. Prado, M. A. Prado, F. Bellivier, C. B. Eap, S. Crettol, P. Vanhoutte, J. Caboche, A. Gratton, L. Moquin, B. Giros, R. Maldonado, S. Daumas, M. Mameli, S. Jamain and S. El Mestikawy (2015). "The absence of VGLUT3 predisposes to cocaine abuse by increasing dopamine and glutamate signaling in the nucleus accumbens." Mol Psychiatry **20**(11): 1448-1459.

Sammeth, M., S. Foissac and R. Guigo (2008). "A general definition and nomenclature for alternative splicing events." PLoS Comput Biol **4**(8): e1000147.

Sandberg, R., J. R. Neilson, A. Sarma, P. A. Sharp and C. B. Burge (2008). "Proliferating cells express mRNAs with shortened 3' untranslated regions and fewer microRNA target sites." Science **320**(5883): 1643-1647.

Santos, M. S., J. Barbosa, Jr., G. S. Veloso, F. Ribeiro, C. Kushmerick, M. V. Gomez, S. S. Ferguson, V. F. Prado and M. A. Prado (2001). "Trafficking of green fluorescent

protein tagged-vesicular acetylcholine transporter to varicosities in a cholinergic cell line." J Neurochem **78**(5): 1104-1113.
Sarter, M., W. J. Gehring and R. Kozak (2006). "More attention must be paid: the neurobiology of attentional effort." Brain Res Rev **51**(2): 145-160.
Sarter, M., M. E. Hasselmo, J. P. Bruno and B. Givens (2005). "Unraveling the attentional functions of cortical cholinergic inputs: interactions between signal-driven and cognitive modulation of signal detection." Brain Res Brain Res Rev **48**(1): 98-111.
Sarter, M., V. Parikh and W. M. Howe (2009). "Phasic acetylcholine release and the volume transmission hypothesis: time to move on." Nat Rev Neurosci **10**(5): 383-390.
Saunders, A., A. J. Granger and B. L. Sabatini (2015). "Corelease of acetylcholine and GABA from cholinergic forebrain neurons." Elife **4**.
Seeger, T., I. Fedorova, F. Zheng, T. Miyakawa, E. Koustova, J. Gomeza, A. S. Basile, C. Alzheimer and J. Wess (2004). "M2 muscarinic acetylcholine receptor knock-out mice show deficits in behavioral flexibility, working memory, and hippocampal plasticity." J Neurosci **24**(45): 10117-10127.
Seguela, P., J. Wadiche, K. Dineley-Miller, J. A. Dani and J. W. Patrick (1993). "Molecular cloning, functional properties, and distribution of rat brain alpha 7: a nicotinic cation channel highly permeable to calcium." J Neurosci **13**(2): 596-604.
Selkoe, D. J. (1993). "Physiological production of the beta-amyloid protein and the mechanism of Alzheimer's disease." Trends Neurosci **16**(10): 403-409.
Selkoe, D. J. (2002). "Alzheimer's disease is a synaptic failure." Science **298**(5594): 789-791.
Semba, K. (2004). "Phylogenetic and ontogenetic aspects of the basal forebrain cholinergic neurons and their innervation of the cerebral cortex." Prog Brain Res **145**: 3-43.
Sengupta, A., J. Kabat, M. Novak, Q. Wu, I. Grundke-Iqbal and K. Iqbal (1998). "Phosphorylation of tau at both Thr 231 and Ser 262 is required for maximal inhibition of its binding to microtubules." Arch Biochem Biophys **357**(2): 299-309.
Seraphin, B. and M. Rosbash (1989). "Identification of functional U1 snRNA-pre-mRNA complexes committed to spliceosome assembly and splicing." Cell **59**(2): 349-358.

Shen, W., S. E. Hamilton, N. M. Nathanson and D. J. Surmeier (2005). "Cholinergic suppression of KCNQ channel currents enhances excitability of striatal medium spiny neurons." J Neurosci **25**(32): 7449-7458.

Shirey, J. K., A. E. Brady, P. J. Jones, A. A. Davis, T. M. Bridges, J. P. Kennedy, S. B. Jadhav, U. N. Menon, Z. Xiang, M. L. Watson, E. P. Christian, J. J. Doherty, M. C. Quirk, D. H. Snyder, J. J. Lah, A. I. Levey, M. M. Nicolle, C. W. Lindsley and P. J. Conn (2009). "A selective allosteric potentiator of the M1 muscarinic acetylcholine receptor increases activity of medial prefrontal cortical neurons and restores impairments in reversal learning." J Neurosci **29**(45): 14271-14286.

Simon, J. R. and M. G. Kuhar (1975). "Impulse-flow regulation of high affinity choline uptake in brain cholinergic nerve terminals." Nature **255**(5504): 162-163.

Sisodia, S. S., E. H. Koo, K. Beyreuther, A. Unterbeck and D. L. Price (1990). "Evidence that beta-amyloid protein in Alzheimer's disease is not derived by normal processing." Science **248**(4954): 492-495.

Smith, C. W. and B. Nadal-Ginard (1989). "Mutually exclusive splicing of alpha-tropomyosin exons enforced by an unusual lariat branch point location: implications for constitutive splicing." Cell **56**(5): 749-758.

Sondag, C. M., G. Dhawan and C. K. Combs (2009). "Beta amyloid oligomers and fibrils stimulate differential activation of primary microglia." J Neuroinflammation **6**: 1.

Song, J. S. and S. D. Yang (1995). "Tau protein kinase I/GSK-3 beta/kinase FA in heparin phosphorylates tau on Ser199, Thr231, Ser235, Ser262, Ser369, and Ser400 sites phosphorylated in Alzheimer disease brain." J Protein Chem **14**(2): 95-105.

Soreq, H. and S. Seidman (2001). "Acetylcholinesterase--new roles for an old actor." Nat Rev Neurosci **2**(4): 294-302.

Squire, L. R. (1992). "Memory and the hippocampus: a synthesis from findings with rats, monkeys, and humans." Psychol Rev **99**(2): 195-231.

Srinivasan, M., C. F. Edman and H. Schulman (1994). "Alternative splicing introduces a nuclear localization signal that targets multifunctional CaM kinase to the nucleus." J Cell Biol **126**(4): 839-852.

Stamm, S., S. Ben-Ari, I. Rafalska, Y. Tang, Z. Zhang, D. Toiber, T. A. Thanaraj and H. Soreq (2005). "Function of alternative splicing." Gene **344**: 1-20.

Starke, K., M. Gothert and H. Kilbinger (1989). "Modulation of neurotransmitter release by presynaptic autoreceptors." Physiol Rev **69**(3): 864-989.

Steiner, B., E. M. Mandelkow, J. Biernat, N. Gustke, H. E. Meyer, B. Schmidt, G. Mieskes, H. D. Soling, D. Drechsel, M. W. Kirschner and et al. (1990). "Phosphorylation of microtubule-associated protein tau: identification of the site for Ca2(+)-calmodulin dependent kinase and relationship with tau phosphorylation in Alzheimer tangles." EMBO J **9**(11): 3539-3544.

Stevens, C. F. (1993). "Quantal release of neurotransmitter and long-term potentiation." Cell **72 Suppl**: 55-63.

Stockley, J. H. and C. O'Neill (2007). "The proteins BACE1 and BACE2 and beta-secretase activity in normal and Alzheimer's disease brain." Biochem Soc Trans **35**(Pt 3): 574-576.

Strauss, W. L., R. R. Kemper, P. Jayakar, C. F. Kong, L. B. Hersh, D. C. Hilt and M. Rabin (1991). "Human choline acetyltransferase gene maps to region 10q11-q22.2 by in situ hybridization." Genomics **9**(2): 396-398.

Strong, M. J. (2010). "The evidence for altered RNA metabolism in amyotrophic lateral sclerosis (ALS)." J Neurol Sci **288**(1-2): 1-12.

Tacke, R., Y. Chen and J. L. Manley (1997). "Sequence-specific RNA binding by an SR protein requires RS domain phosphorylation: creation of an SRp40-specific splicing enhancer." Proc Natl Acad Sci U S A **94**(4): 1148-1153.

Takashima, A., K. Noguchi, K. Sato, T. Hoshino and K. Imahori (1993). "Tau protein kinase I is essential for amyloid beta-protein-induced neurotoxicity." Proc Natl Acad Sci U S A **90**(16): 7789-7793.

Takimoto, M., T. Tomonaga, M. Matunis, M. Avigan, H. Krutzsch, G. Dreyfuss and D. Levens (1993). "Specific binding of heterogeneous ribonucleoprotein particle protein K to the human c-myc promoter, in vitro." J Biol Chem **268**(24): 18249-18258.

Tan, P. K., C. Waites, Y. Liu, D. E. Krantz and R. H. Edwards (1998). "A leucine-based motif mediates the endocytosis of vesicular monoamine and acetylcholine transporters." J Biol Chem **273**(28): 17351-17360.

Tanaka, C., H. Fujiwara and Y. Fujii (1986). "Acetylcholine release from guinea pig caudate slices evoked by phorbol ester and calcium." FEBS Lett **195**(1-2): 129-134.

Tekin, S. and J. L. Cummings (2002). "Frontal-subcortical neuronal circuits and clinical neuropsychiatry: an update." J Psychosom Res **53**(2): 647-654.

Terry, A. V., Jr. and J. J. Buccafusco (2003). "The cholinergic hypothesis of age and Alzheimer's disease-related cognitive deficits: recent challenges and their implications for novel drug development." J Pharmacol Exp Ther **306**(3): 821-827.

Texido, L., M. Martin-Satue, E. Alberdi, C. Solsona and C. Matute (2011). "Amyloid beta peptide oligomers directly activate NMDA receptors." Cell Calcium **49**(3): 184-190.

Thomsen, M., D. P. Woldbye, G. Wortwein, A. Fink-Jensen, J. Wess and S. B. Caine (2005). "Reduced cocaine self-administration in muscarinic M5 acetylcholine receptor-deficient mice." J Neurosci **25**(36): 8141-8149.

Tollervey, J. R., Z. Wang, T. Hortobagyi, J. T. Witten, K. Zarnack, M. Kayikci, T. A. Clark, A. C. Schweitzer, G. Rot, T. Curk, B. Zupan, B. Rogelj, C. E. Shaw and J. Ule (2011). "Analysis of alternative splicing associated with aging and neurodegeneration in the human brain." Genome Res **21**(10): 1572-1582.

Tomic, J. L., A. Pensalfini, E. Head and C. G. Glabe (2009). "Soluble fibrillar oligomer levels are elevated in Alzheimer's disease brain and correlate with cognitive dysfunction." Neurobiol Dis **35**(3): 352-358.

Torres, G. E., R. R. Gainetdinov and M. G. Caron (2003). "Plasma membrane monoamine transporters: structure, regulation and function." Nat Rev Neurosci **4**(1): 13-25.

Torroja, L., H. Chu, I. Kotovsky and K. White (1999). "Neuronal overexpression of APPL, the Drosophila homologue of the amyloid precursor protein (APP), disrupts axonal transport." Curr Biol **9**(9): 489-492.

Traiffort, E., M. Ruat, S. O'Regan and F. M. Meunier (2005). "Molecular characterization of the family of choline transporter-like proteins and their splice variants." J Neurochem **92**(5): 1116-1125.

Trapnell, C., L. Pachter and S. L. Salzberg (2009). "TopHat: discovering splice junctions with RNA-Seq." Bioinformatics **25**(9): 1105-1111.

Tseng, B. P., K. N. Green, J. L. Chan, M. Blurton-Jones and F. M. LaFerla (2008). "Abeta inhibits the proteasome and enhances amyloid and tau accumulation." Neurobiol Aging **29**(11): 1607-1618.

Turchi, J. and M. Sarter (1997). "Cortical acetylcholine and processing capacity: effects of cortical cholinergic deafferentation on crossmodal divided attention in rats." Brain Res Cogn Brain Res **6**(2): 147-158.

Turner, T. J. (2004). "Nicotine enhancement of dopamine release by a calcium-dependent increase in the size of the readily releasable pool of synaptic vesicles." J Neurosci **24**(50): 11328-11336.

Uhlhaas, P. J. and W. Singer (2006). "Neural synchrony in brain disorders: relevance for cognitive dysfunctions and pathophysiology." Neuron **52**(1): 155-168.

Van der Kloot, W. (2003). "Loading and recycling of synaptic vesicles in the Torpedo electric organ and the vertebrate neuromuscular junction." Prog Neurobiol **71**(4): 269-303.

Van der Kloot, W. and J. Molgo (1994). "Quantal acetylcholine release at the vertebrate neuromuscular junction." Physiol Rev **74**(4): 899-991.

Van der Ploeg, L. H., A. Y. Liu, P. A. Michels, T. De Lange, P. Borst, H. K. Majumder, H. Weber, G. H. Veeneman and J. Van Boom (1982). "RNA splicing is required to make the messenger RNA for a variant surface antigen in trypanosomes." Nucleic Acids Res **10**(12): 3591-3604.

Van Dort, C. J., D. P. Zachs, J. D. Kenny, S. Zheng, R. R. Goldblum, N. A. Gelwan, D. M. Ramos, M. A. Nolan, K. Wang, F. J. Weng, Y. Lin, M. A. Wilson and E. N. Brown (2015). "Optogenetic activation of cholinergic neurons in the PPT or LDT induces REM sleep." Proc Natl Acad Sci U S A **112**(2): 584-589.

Vana, L., N. M. Kanaan, I. C. Ugwu, J. Wuu, E. J. Mufson and L. I. Binder (2011). "Progression of tau pathology in cholinergic Basal forebrain neurons in mild cognitive impairment and Alzheimer's disease." Am J Pathol **179**(5): 2533-2550.

Vardy, E., I. T. Arkin, K. E. Gottschalk, H. R. Kaback and S. Schuldiner (2004). "Structural conservation in the major facilitator superfamily as revealed by comparative modeling." Protein Sci **13**(7): 1832-1840.

Varoqui, H. and J. D. Erickson (1996). "Active transport of acetylcholine by the human vesicular acetylcholine transporter." J Biol Chem **271**(44): 27229-27232.

Vassar, R. (2001). "The beta-secretase, BACE: a prime drug target for Alzheimer's disease." J Mol Neurosci **17**(2): 157-170.

Vilaro, M. T., J. M. Palacios and G. Mengod (1990). "Localization of m5 muscarinic receptor mRNA in rat brain examined by in situ hybridization histochemistry." Neurosci Lett **114**(2): 154-159.

Villarroya-Beltri, C., C. Gutierrez-Vazquez, F. Sanchez-Cabo, D. Perez-Hernandez, J. Vazquez, N. Martin-Cofreces, D. J. Martinez-Herrera, A. Pascual-Montano, M. Mittelbrunn and F. Sanchez-Madrid (2013). "Sumoylated hnRNPA2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs." Nat Commun **4**: 2980.

Visa, N., A. T. Alzhanova-Ericsson, X. Sun, E. Kiseleva, B. Bjorkroth, T. Wurtz and B. Daneholt (1996). "A pre-mRNA-binding protein accompanies the RNA from the gene through the nuclear pores and into polysomes." Cell **84**(2): 253-264.

Vuong, C. K., D. L. Black and S. Zheng (2016). "The neurogenetics of alternative splicing." Nat Rev Neurosci **17**(5): 265-281.

Wada, E., K. Wada, J. Boulter, E. Deneris, S. Heinemann, J. Patrick and L. W. Swanson (1989). "Distribution of alpha 2, alpha 3, alpha 4, and beta 2 neuronal nicotinic receptor subunit mRNAs in the central nervous system: a hybridization histochemical study in the rat." J Comp Neurol **284**(2): 314-335.

Wall, S. J., R. P. Yasuda, M. Li and B. B. Wolfe (1991). "Development of an antiserum against m3 muscarinic receptors: distribution of m3 receptors in rat tissues and clonal cell lines." Mol Pharmacol **40**(5): 783-789.

Wallace, T. L., P. M. Callahan, A. Tehim, D. Bertrand, G. Tombaugh, S. Wang, W. Xie, W. B. Rowe, V. Ong, E. Graham, A. V. Terry, Jr., J. S. Rodefer, B. Herbert, M. Murray, R. Porter, L. Santarelli and D. A. Lowe (2011). "RG3487, a novel nicotinic alpha7 receptor partial agonist, improves cognition and sensorimotor gating in rodents." J Pharmacol Exp Ther **336**(1): 242-253.

Walsh, D. M., I. Klyubin, J. V. Fadeeva, W. K. Cullen, R. Anwyl, M. S. Wolfe, M. J. Rowan and D. J. Selkoe (2002). "Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo." Nature **416**(6880): 535-539.

Wang, H., M. Yu, M. Ochani, C. A. Amella, M. Tanovic, S. Susarla, J. H. Li, H. Wang, H. Yang, L. Ulloa, Y. Al-Abed, C. J. Czura and K. J. Tracey (2003). "Nicotinic acetylcholine

receptor alpha7 subunit is an essential regulator of inflammation." Nature **421**(6921): 384-388.

Wang, H. W., J. F. Pasternak, H. Kuo, H. Ristic, M. P. Lambert, B. Chromy, K. L. Viola, W. L. Klein, W. B. Stine, G. A. Krafft and B. L. Trommer (2002). "Soluble oligomers of beta amyloid (1-42) inhibit long-term potentiation but not long-term depression in rat dentate gyrus." Brain Res **924**(2): 133-140.

Wang, H. Y., D. H. Lee, M. R. D'Andrea, P. A. Peterson, R. P. Shank and A. B. Reitz (2000). "beta-Amyloid(1-42) binds to alpha7 nicotinic acetylcholine receptor with high affinity. Implications for Alzheimer's disease pathology." J Biol Chem **275**(8): 5626-5632.

Wang, P., G. Yang, D. R. Mosier, P. Chang, T. Zaidi, Y. D. Gong, N. M. Zhao, B. Dominguez, K. F. Lee, W. B. Gan and H. Zheng (2005). "Defective neuromuscular synapses in mice lacking amyloid precursor protein (APP) and APP-Like protein 2." J Neurosci **25**(5): 1219-1225.

Wei, J., E. A. Walton, A. Milici and J. J. Buccafusco (1994). "m1-m5 muscarinic receptor distribution in rat CNS by RT-PCR and HPLC." J Neurochem **63**(3): 815-821.

Weidemann, A., G. Konig, D. Bunke, P. Fischer, J. M. Salbaum, C. L. Masters and K. Beyreuther (1989). "Identification, biogenesis, and localization of precursors of Alzheimer's disease A4 amyloid protein." Cell **57**(1): 115-126.

Weihe, E., J. H. Tao-Cheng, M. K. Schafer, J. D. Erickson and L. E. Eiden (1996). "Visualization of the vesicular acetylcholine transporter in cholinergic nerve terminals and its targeting to a specific population of small synaptic vesicles." Proc Natl Acad Sci U S A **93**(8): 3547-3552.

Wesnes, K. and D. M. Warburton (1984). "Effects of scopolamine and nicotine on human rapid information processing performance." Psychopharmacology (Berl) **82**(3): 147-150.

Wess, J. (1996). "Molecular biology of muscarinic acetylcholine receptors." Crit Rev Neurobiol **10**(1): 69-99.

Wess, J. (2004). "Muscarinic acetylcholine receptor knockout mice: novel phenotypes and clinical implications." Annu Rev Pharmacol Toxicol **44**: 423-450.

Wevers, A., A. Jeske, C. Lobron, C. Birtsch, S. Heinemann, A. Maelicke, R. Schroder and H. Schroder (1994). "Cellular distribution of nicotinic acetylcholine receptor subunit mRNAs in the human cerebral cortex as revealed by non-isotopic in situ hybridization." Brain Res Mol Brain Res **25**(1-2): 122-128.

White, J. A., A. M. Manelli, K. H. Holmberg, L. J. Van Eldik and M. J. Ladu (2005). "Differential effects of oligomeric and fibrillar amyloid-beta 1-42 on astrocyte-mediated inflammation." Neurobiol Dis **18**(3): 459-465.

Whitehouse, P. J., D. L. Price, R. G. Struble, A. W. Clark, J. T. Coyle and M. R. Delon (1982). "Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain." Science **215**(4537): 1237-1239.

Whitton, P. S., I. G. Marshall and S. M. Parsons (1986). "Reduction of quantal size by vesamicol (AH5183), an inhibitor of vesicular acetylcholine storage." Brain Res **385**(1): 189-192.

Winitz, S., M. Russell, N. X. Qian, A. Gardner, L. Dwyer and G. L. Johnson (1993). "Involvement of Ras and Raf in the Gi-coupled acetylcholine muscarinic m2 receptor activation of mitogen-activated protein (MAP) kinase kinase and MAP kinase." J Biol Chem **268**(26): 19196-19199.

Wink, A. M., F. Bernard, R. Salvador, E. Bullmore and J. Suckling (2006). "Age and cholinergic effects on hemodynamics and functional coherence of human hippocampus." Neurobiol Aging **27**(10): 1395-1404.

Woehrling, E. K., H. R. Parri, E. H. Tse, E. J. Hill, I. D. Maidment, G. C. Fox and M. D. Coleman (2015). "A predictive in vitro model of the impact of drugs with anticholinergic properties on human neuronal and astrocytic systems." PLoS One **10**(3): e0118786.

Wolfe, B. B. and R. P. Yasuda (1995). "Development of selective antisera for muscarinic cholinergic receptor subtypes." Ann N Y Acad Sci **757**: 186-193.

Woolf, N. J. and L. L. Butcher (1981). "Cholinergic neurons in the caudate-putamen complex proper are intrinsically organized: a combined Evans blue and acetylcholinesterase analysis." Brain Res Bull **7**(5): 487-507.

Wrenn, C. C., D. A. Lappi and R. G. Wiley (1999). "Threshold relationship between lesion extent of the cholinergic basal forebrain in the rat and working memory impairment in the radial maze." Brain Res **847**(2): 284-298.

Wu, C. F., R. Bertorelli, M. Sacconi, G. Pepeu and S. Consolo (1988). "Decrease of brain acetylcholine release in aging freely-moving rats detected by microdialysis." Neurobiol Aging **9**(4): 357-361.

Wu, J. Y. and T. Maniatis (1993). "Specific interactions between proteins implicated in splice site selection and regulated alternative splicing." Cell **75**(6): 1061-1070.

Xia, D., R. J. Kelleher, 3rd and J. Shen (2016). "Loss of Abeta43 Production Caused by Presenilin-1 Mutations in the Knockin Mouse Brain." Neuron **90**(2): 417-422.

Xiao, S. H. and J. L. Manley (1997). "Phosphorylation of the ASF/SF2 RS domain affects both protein-protein and protein-RNA interactions and is necessary for splicing." Genes Dev **11**(3): 334-344.

Yakel, J. L. (2012). "Nicotinic ACh receptors in the hippocampus: role in excitability and plasticity." Nicotine Tob Res **14**(11): 1249-1257.

Yakel, J. L. and Z. Shao (2004). "Functional and molecular characterization of neuronal nicotinic ACh receptors in rat hippocampal interneurons." Prog Brain Res **145**: 95-107.

Yamada, M., T. Miyakawa, A. Duttaroy, A. Yamanaka, T. Moriguchi, R. Makita, M. Ogawa, C. J. Chou, B. Xia, J. N. Crawley, C. C. Felder, C. X. Deng and J. Wess (2001). "Mice lacking the M3 muscarinic acetylcholine receptor are hypophagic and lean." Nature **410**(6825): 207-212.

Yamamura, H. I. and S. H. Snyder (1972). "Choline: high-affinity uptake by rat brain synaptosomes." Science **178**(4061): 626-628.

Yan, R., M. J. Bienkowski, M. E. Shuck, H. Miao, M. C. Tory, A. M. Pauley, J. R. Brashier, N. C. Stratman, W. R. Mathews, A. E. Buhl, D. B. Carter, A. G. Tomasselli, L. A. Parodi, R. L. Heinrikson and M. E. Gurney (1999). "Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity." Nature **402**(6761): 533-537.

Yan, Z., J. Flores-Hernandez and D. J. Surmeier (2001). "Coordinated expression of muscarinic receptor messenger RNAs in striatal medium spiny neurons." Neuroscience **103**(4): 1017-1024.

Yang, Y., C. D. Paspalas, L. E. Jin, M. R. Picciotto, A. F. Arnsten and M. Wang (2013). "Nicotinic alpha7 receptors enhance NMDA cognitive circuits in dorsolateral prefrontal cortex." Proc Natl Acad Sci U S A **110**(29): 12078-12083.

Yeomans, J., G. Forster and C. Blaha (2001). "M5 muscarinic receptors are needed for slow activation of dopamine neurons and for rewarding brain stimulation." Life Sci **68**(22-23): 2449-2456.

You, Z. B., B. Wang, D. Zitzman and R. A. Wise (2008). "Acetylcholine release in the mesocorticolimbic dopamine system during cocaine seeking: conditioned and unconditioned contributions to reward and motivation." J Neurosci **28**(36): 9021-9029.

Young, J. W., N. Crawford, J. S. Kelly, L. E. Kerr, H. M. Marston, C. Spratt, K. Finlayson and J. Sharkey (2007). "Impaired attention is central to the cognitive deficits observed in alpha 7 deficient mice." Eur Neuropsychopharmacol **17**(2): 145-155.

Yu, G., M. Nishimura, S. Arawaka, D. Levitan, L. Zhang, A. Tandon, Y. Q. Song, E. Rogaeva, F. Chen, T. Kawarai, A. Supala, L. Levesque, H. Yu, D. S. Yang, E. Holmes, P. Milman, Y. Liang, D. M. Zhang, D. H. Xu, C. Sato, E. Rogaev, M. Smith, C. Janus, Y. Zhang, R. Aebersold, L. S. Farrer, S. Sorbi, A. Bruni, P. Fraser and P. St George-Hyslop (2000). "Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and betaAPP processing." Nature **407**(6800): 48-54.

Zaborszky, L. (1989). "Afferent connections of the forebrain cholinergic projection neurons, with special reference to monoaminergic and peptidergic fibers." EXS **57**: 12-32.

Zaborszky, L. and A. Duque (2000). "Local synaptic connections of basal forebrain neurons." Behav Brain Res **115**(2): 143-158.

Zaborszky, L., K. Pang, J. Somogyi, Z. Nadasdy and I. Kallo (1999). "The basal forebrain corticopetal system revisited." Ann N Y Acad Sci **877**: 339-367.

Zachariou, V., B. J. Caldarone, A. Weathers-Lowin, T. P. George, J. D. Elsworth, R. H. Roth, J. P. Changeux and M. R. Picciotto (2001). "Nicotine receptor inactivation decreases sensitivity to cocaine." Neuropsychopharmacology **24**(5): 576-589.

Zahler, A. M., K. M. Neugebauer, W. S. Lane and M. B. Roth (1993). "Distinct functions of SR proteins in alternative pre-mRNA splicing." Science **260**(5105): 219-222.

Zahm, D. S. and M. Trimble (2008). "The dopaminergic projection system, basal forebrain macrosystems, and conditioned stimuli." CNS Spectr **13**(1): 32-40.

Zhang, B., A. Maiti, S. Shively, F. Lakhani, G. McDonald-Jones, J. Bruce, E. B. Lee, S. X. Xie, S. Joyce, C. Li, P. M. Toleikis, V. M. Lee and J. Q. Trojanowski (2005).

"Microtubule-binding drugs offset tau sequestration by stabilizing microtubules and reversing fast axonal transport deficits in a tauopathy model." Proc Natl Acad Sci U S A **102**(1): 227-231.

Zhang, W., M. Yamada, J. Gomeza, A. S. Basile and J. Wess (2002). "Multiple muscarinic acetylcholine receptor subtypes modulate striatal dopamine release, as studied with M1-M5 muscarinic receptor knock-out mice." J Neurosci **22**(15): 6347-6352.

Zhang, Y., L. Chen, G. Shen, Q. Zhao, L. Shangguan and M. He (2014). "GRK5 dysfunction accelerates tau hyperphosphorylation in APP (swe) mice through impaired cholinergic activity." Neuroreport **25**(7): 542-547.

Zhang, Y., R. McLaughlin, C. Goodyer and A. LeBlanc (2002). "Selective cytotoxicity of intracellular amyloid beta peptide1-42 through p53 and Bax in cultured primary human neurons." J Cell Biol **156**(3): 519-529.

Zhao, W. Q., F. G. De Felice, S. Fernandez, H. Chen, M. P. Lambert, M. J. Quon, G. A. Krafft and W. L. Klein (2008). "Amyloid beta oligomers induce impairment of neuronal insulin receptors." FASEB J **22**(1): 246-260.

Zheng, H., M. Jiang, M. E. Trumbauer, R. Hopkins, D. J. Sirinathsinghji, K. A. Stevens, M. W. Conner, H. H. Slunt, S. S. Sisodia, H. Y. Chen and L. H. Van der Ploeg (1996). "Mice deficient for the amyloid precursor protein gene." Ann N Y Acad Sci **777**: 421-426.

Zheng, W. H., S. Bastianetto, F. Mennicken, W. Ma and S. Kar (2002). "Amyloid beta peptide induces tau phosphorylation and loss of cholinergic neurons in rat primary septal cultures." Neuroscience **115**(1): 201-211.

Zhou, F. M., C. J. Wilson and J. A. Dani (2002). "Cholinergic interneuron characteristics and nicotinic properties in the striatum." J Neurobiol **53**(4): 590-605.

Zhou, H., A. Meyer, K. Starke, J. Gomeza, J. Wess and A. U. Trendelenburg (2002). "Heterogeneity of release-inhibiting muscarinic autoreceptors in heart atria and urinary bladder: a study with M(2)- and M(4)-receptor-deficient mice." Naunyn Schmiedebergs Arch Pharmacol **365**(2): 112-122.

Zoli, M., M. R. Picciotto, R. Ferrari, D. Cocchi and J. P. Changeux (1999). "Increased neurodegeneration during ageing in mice lacking high-affinity nicotine receptors." EMBO J **18**(5): 1235-1244.

Chapter 2

ChAT–ChR2–EYFP mice have enhanced motor endurance but show deficits in attention and several additional cognitive domains

2.1 Chapter Summary

Acetylcholine (ACh) is an important neuromodulator in the nervous system implicated in many forms of cognitive and motor processing. Recent studies have used bacterial artificial chromosome (BAC) transgenic mice expressing channelrhodopsin-2 (ChR2) protein under the control of the choline acetyltransferase (ChAT) promoter (ChAT–ChR2–EYFP) to dissect cholinergic circuit connectivity and function using optogenetic approaches. We report that a mouse line used for this purpose also carries several copies of the vesicular acetylcholine transporter gene (VAChT), which leads to overexpression of functional VAChT and consequently increased cholinergic tone. We demonstrate that these mice have marked improvement in motor endurance. However, they also present severe cognitive deficits, including attention deficits and dysfunction in working memory and spatial memory. These results suggest that increased VAChT expression may disrupt critical steps in information processing. Our studies demonstrate that ChAT–ChR2–EYFP mice show altered cholinergic tone that fundamentally differentiates them from wild-type mice.

2.2 Introduction

Acetylcholine (ACh) has multiple functions in the CNS, including modulation of attention and memory encoding, consolidation, and retrieval (Prado et al., 2013). Understanding the precise roles of ACh in distinct brain regions has remained a challenge because of the myriad of cholinergic receptors that can modulate postsynaptic and presynaptic cholinergic activities (Hasselmo and Sarter, 2011). Genetic approaches have been used to determine the specific roles of ACh in different brain regions (Guzman et al., 2011; Martyn et al., 2012; Patel et al., 2012), as well as to pinpoint precise functions of ACh receptors (for review, see Wess et al., 2007; Changeux, 2010). More recently, optogenetics has also been used to further dissect and understand cholinergic signaling in the CNS (Witten et al., 2010; Nagode et al., 2011; Ren et al., 2011; Zhao et al., 2011; Gu et al., 2012). One of the approaches to specifically activate cholinergic neurons using optogenetics is in vivo injection of Cre-inducible viral vectors carrying the channelrhodopsin-2 (ChR2) gene. Cholinergic specificity is ensured by using bacterial artificial chromosome (BAC) transgenic mice expressing Cre recombinase under the control of the choline acetyltransferase (ChAT) promoter (Gradinaru et al., 2007). Alternatively, BAC transgenic mice expressing ChR2 protein under the control of the ChAT promoter (ChAT–ChR2–EYFP) have also been used, for example, to examine ACh/glutamate cotransmission in neurons thought to be strictly cholinergic (Ren et al., 2011; Zhao et al., 2011). However, a unique characteristic of the ChAT locus, also called cholinergic gene locus (Eiden, 1998), is that the entire open reading frame for the vesicular acetylcholine transporter (VAChT) lies within the intron between the first and second exons of the ChAT gene (Bejanin et al., 1994; Erickson et al., 1994; Roghani et al., 1994; Cervini et al., 1995; Naciff et al., 1997). Thus, the BAC containing the ChAT gene used to generate these mouse lines carries also the VAChT gene.

Importantly, increased expression of VAChT can alter ACh release. For example, in vitro overexpression of the VAChT in Xenopus neurons results in increased amplitude of miniature currents and in more synaptic vesicles containing ACh (Song et al., 1997). Moreover, a recent report indicates that a mouse line containing four copies of the ChAT–BAC driving the expression of GFP presents increased ACh release (Nagy and

Aubert, 2012). However, the consequences of VAChT overexpression for cognitive functions are not yet understood. Because ChAT–ChR2–EYFP mice have the potential to become widely used by the neuroscience community as a tool to determine the consequences of cholinergic activation for behavior manifestations, we determined the extent by which VAChT is overexpressed in this mouse line and whether overexpression of VAChT affects mouse behavior.

We report that ChAT–ChR2–EYFP mice have several extra copies of the VAChT gene and express increased VAChT mRNA and protein levels. ACh release is increased threefold in these mice. Importantly, we find that ChAT–ChR2–EYFP have increased physical endurance, consistent with increased cholinergic tone. In contrast with the improved motor function, cognitive tests demonstrated that increased expression of VAChT interferes with multiple domains of cognitive function.

2.3 Material and Methods

2.3.1 Animals

All experiments were performed in compliance with the Canadian Council of Animal Care guidelines for the care and use of animals. The protocol was approved by the University of Western Ontario Institutional Animal Care and Use Committee (2008-127). All efforts were made to minimize the suffering of animals. ChAT–ChR2–EYFP mice [B6.Cg-Tg(Chat-COP4*H134R/EYFP)6Gfng/J; The Jackson Laboratory] and VGAT–ChR2–EYFP mice [B6.Cg-Tg(Slc32a1-COP4*H134R/EYFP)8Gfng/J; The Jackson Laboratory] were described previously (Zhao et al., 2011) and were maintained as hemizygous. Control mice consisted of ChAT–ChR2–EYFP or VGAT–ChR2–EYFP negative littermates. Only male mice were used in these studies. Animals were housed in groups of two to four per cage in a temperature-controlled room with a 14/10 light/dark cycle. Food and water were provided ad libitum. Behavioral assessment started with less demanding (locomotor activity) to more demanding (depression and anxiety-like behavior, spatial memory in the Barnes maze, water maze, and then attention) tasks. Treadmill experiments were done after the water maze and before

attention measurements. There was an interval of 3–5 d between distinct behavioral tasks. The experimenter was blind to the genotypes, and, in most behavioral tasks, software-based analysis was used to score mouse performance. All behavioral experiments were performed from 9:00 A.M. to 4:00 P.M. in the light cycle, except for the light/dark transition (always performed after 7:00 P.M.) and locomotor activity tests (performed from 5:00 P.M. to 9:00 P.M.; lights off at 7:00 P.M.).

2.3.2 Immunofluorescence microscopy

Mice were anesthetized using a ketamine (100 mg/kg)-xylene (20 mg/kg) solution and then killed by transcardial perfusion with 4% paraformaldehyde (v/v) in 1× PBS. Brains were harvested and placed in 4% paraformaldehyde in 1× PBS at 4°C for 4 h, and they were kept at 4°C until being sliced using a vibratome. Brain sections (40 µm) were prepared, and free-floating sections in 1× PBS (one per well in a 24-well plate) were permeabilized with 0.4% Triton X-100 in 1× PBS for 1 h. Nonspecific epitopes were blocked using a solution of 1× PBS/0.4% Triton X-100 containing 0.1% glycine (w/v), 0.1% lysine (w/v), 1% BSA (w/v), and 1% normal donkey serum (w/v). Primary antibody (an FITC-conjugated goat polyclonal anti-GFP; catalog #ab6662; Abcam) was incubated in blocking buffer overnight at 4°C. Sections were then washed five times in 1× PBS/0.4% Triton X-100 (10 min each). Sections were mounted on slides and visualized using an Olympus IX81 laser-scanning microscope (FluoView) using an argon laser with parameters set for GFP. Images were taken using a 10× objective (numerical aperture 0.40) with the tile feature. Acquired images were then used to reconstruct the entire brain using the Olympus software.

2.3.3 qPCR and Western blotting

To genotype mice and to measure gene copies of VAChT, genomic DNA was extracted from tail-snip samples, and qPCR was used with the following primer pair: forward, 5′-GAGAGTACTTTGCCTGGGAGGA-3′; and reverse, 5′-GGCCACAGTAAGACCTCCCTTG-3′. The results were normalized to Sti1p1 using the following primer pair: forward, 5′-ATGTATCTGAGCATGCCTCTG-3′; and reverse, 5′-

ATTGCCCTTCTCCTTTAGCTC-3′. To measure VAChT mRNA expression, total RNA was extracted using the Aurum Total RNA for fatty and fibrous tissue kit (Bio-Rad) according to the kit manual. cDNA synthesis and qPCR analysis were performed as described previously (Guzman et al., 2011). Immunoblotting was performed as described previously (Martins-Silva et al., 2011). The antibodies used were anti-VAChT (catalog #139103; Synaptic Systems), anti-ChAT (catalog #A144p; Millipore), anti-Synaptophysin (catalog #S5768; Sigma-Aldrich), and anti-Actin (catalog #ab49900; Abcam).

2.3.4 Acetylcholine Release

ACh release from hippocampal brain slices was done as described previously (Guzman et al., 2011), by labeling slices with [3H] methyl-choline, before using KCl to stimulate release of labeled ACh.

2.3.5 Metabolic assessments

Oxygen consumption, carbon dioxide production, respiratory exchange ratio (RER), food and water intake, and physical activity were simultaneously measured for young and adult mice by using the Comprehensive Lab Animal Monitoring System interfaced with Oxymax software (Columbus Instruments) essentially as described previously (Guzman et al., 2013). Mice were individually housed in the metabolic chambers maintained at 24 ± 1°C and given ad libitum access to powdered standard rodent chow and water. All the measurements were taken every 10 min for 24 h (12 h light/12 h dark) after a 16 h habituation period in the individual metabolic chambers. Total activity, ambulatory activity, and sleep (periods of inactivity) were obtained using the Opto-M3 Activity Monitor and Oxymax software algorithms (Columbus Instruments) as described previously (Guzman et al., 2013).

2.3.6 Glucose tolerance test

Animals were fasted for 5 h and then received an intraperitoneal injection of 2 g/kg glucose. Blood glucose levels were measured at 0 (baseline), 30, 60, 90, 120, and 150

min after glucose injection. Glucose levels were determined in blood samples obtained from a tail snip using ACCU-CHEK Advantage (Roche Diagnostics).

2.3.7 Grip force

Forelimb and hindlimb grip strength were assessed using a previously described protocol (Prado et al., 2006).

2.3.8 Treadmill

To test motor endurance, a rodent treadmill (IITC Life Sciences), with a grid behind the track that delivered a mild electric shock (15–20 V) when the mouse fell off, was used. Before testing, mice were trained for 4 d (3 min/day). On the first day, inclination was set to 5°. The inclination was increased by 5° on each subsequent training day. The initial training speed was 8 m/min, and the treadmill was accelerated by 1 m/min, up to 9 m/min. In the second training session, the initial speed was 10 m/min and was increased to 11 m/min, whereas on the third day and fourth days, the speed was maintained at 12 m/min. On the test day, the initial speed was set to 12 m/min, and the ramp angle was set to 20°. Speed was increased to 20 m/min over the course of the first 15 min of testing, after which it remained constant, until the test was complete. The test ended when 60 min had elapsed or the mouse had reached exhaustion (Lund et al., 2010).

2.3.9 Elevated plus maze, forced swimming test, and tail suspension test

Anxiety-like behavior was assessed using the elevated plus maze test, performed as described previously (Martins-Silva et al., 2011). Sessions were recorded and the video was analyzed using the ANY-Maze Software (Stoelting) to determine total time spent in the open and closed arms. Depressive-like behavior was assessed using the forced swim and tail suspension tests (Martyn et al., 2012). For the forced swim test, mice were placed in a 2 L beaker containing 1.8 L of 25–27°C water, for 6 min. For the tail suspension test, mice were suspended from their tails for 5 min, held in place by a strip

of masking tape placed ~1.5 inches from the base of the tail. Sessions were recorded for both tests, and immobility time and episodes were assessed using the ANY-Maze Software. For the forced swim test, only data obtained after the initial 2 min of the test were used for the analysis.

2.3.10 Rotarod

The rotarod task was conducted as described previously to assess motor learning and acrobatic motor skill (Prado et al., 2006; de Castro et al., 2009a).

2.3.11 Locomotor activity

Spontaneous locomotor activity in a new environment to determine exploratory behavior was recorded using automated locomotor boxes essentially as described previously (Guzman et al., 2013).

2.3.12 Spontaneous alternations Y-maze

The spontaneous alternations Y-maze task to investigate working memory was performed using a symmetrical, three-armed Y-maze as described previously (de Castro et al., 2009a). All sessions were recorded. Both the order and the number of arm entries were recorded. A spontaneous alternation was counted when the mouse visited all three arms in a row without revisiting a previous arm.

2.3.13 Barnes maze

Barnes maze testing to determine spatial memory was performed as described previously (Patil et al., 2009; Martyn et al., 2012) using a white circular platform (92 cm in diameter) with 20 equally spaced holes (5 cm in diameter; 7.5 cm between holes), elevated 105 cm above the floor (San Diego Instruments), and spatial cues (posters, streamers, and plastic props) were placed around the maze. Briefly, animals were given four training trials a day for 4 d, with a 15 min intertrial interval (ITI). On the fifth day, memory was assessed via a probe trial. The probe trial consisted of barring access to

the target hole and assessing nose pokes to the holes within the target quadrant. Sessions were recorded and analyzed using the ANY-Maze Software.

2.3.14 Morris water maze

The spatial version of the Morris water maze (MWM) was conducted as described previously to investigate spatial memory (Vorhees and Williams, 2006; Martyn et al., 2012). Briefly, animals were given four training trials a day (90 s each) for 4 d, with a 15 min ITI. If the mice did not find the platform after 90 s during the learning phase, they were gently directed to the platform. On the fifth day, memory was assessed via a probe trial (60 s), during which the platform is removed and time spent in the target quadrant is measured. The task was performed in a 1.5-m-diameter pool with 25°C water. The platform was submerged 1 cm below the surface of the water, and spatial cues (posters, streamers, and plastic props) were distributed around the pool. Sessions were recorded and analyzed using the ANY-Maze Software.

Both the two-trial and cued variations of the MWM were performed as described previously (Vorhees and Williams, 2006). Briefly, for the two-trial variation, used to assess working or trial-dependent learning and memory, mice were tested over the course of 8 d. The mouse was first given a 90 s trial, and then after a 15 s ITI, the mouse was given a second trial with identical platform location and starting point. This was repeated with four unique starting location/platform location combinations a day. As for the cued variation, used to assess goal-directed behavior, mice were tested for 2 d using novel platform and starting location combinations. For this variation, the platform was at water level and a cue was placed on it (a plastic block). Sessions were recorded for both tests and were analyzed using the ANY-Maze Software.

2.3.15 Five-choice serial reaction time task

The five-choice serial reaction time (5-CSRT) task is used to determine attention in mice (Robbins, 2002; Romberg et al., 2011). Mice were trained in the 5-CSRT in automated Bussey-Saksida Mouse Touch Screen Systems model 81426 (Campden Instruments Limited). Schedules were designed and data were collected using the ABET II Touch

software v.2.15 (Lafayette Instruments). Before being trained on the 5-CSRT task, mice were first put through a pretraining program. This consisted of first habituating the mouse to the testing chamber with the lights off for 15 min. The next day, the mouse was left in the chamber with the lights off for 20 min. At this time, the reward tray was primed with strawberry milkshake (Saputo Dairy Products), and a tone was played when the mouse entered the reward tray. Whenever the mouse returned to the reward tray, it received a reward paired with the tone. This was repeated the next 2 d for 40 min sessions (phase 1).

The next training phase (phase 2) involved pairing the reward with presentation of the stimulus (flash of light in one of the five windows) on the touchscreen. The stimulus appeared randomly, and, after 30 s, it was removed and a reward was given paired with a tone. If the mouse touched the screen while stimulus was displayed, it immediately received a reward. Once the mouse collected the reward, a new trial was initiated. This phase was repeated until the mouse completed 30 trials within 60 min (phase 2).

To further shape behavior, phase 3 involved displaying the stimulus randomly in one of the windows. The mouse had to touch the stimulus on the screen to receive a reward paired with a tone. There was no response to the mouse touching anything but the stimulus. Once again, this was repeated until the mouse completed 30 trials within 60 min. The next phase (phase 4) was identical to phase 3 except that the mouse had to initiate each trial by nose poking the reward tray. Criterion was 30 correct trials within 60 min.

Finally, in the last pre-training phase (phase 5), the previous procedure was repeated, but if the mouse touched an incorrect screen, it received a 5 s timeout, during which the chamber light was turned on. The final phase had a stricter criterion, requiring the mice to perform 30 trials in 60 min with 23 correct responses in 2 consecutive days.

For the 5-CSRT training phase, mice were trained to respond to brief flashes of light pseudo randomly displayed in one of the five spatial locations on the touchscreen. Each trial was initiated after the mouse poked the magazine. In this phase, the stimulus was delivered after a variable 5–10 s delay (delay period), during which the animal was required to attend to the screen. In case the mouse prematurely touched the screen

during this delay, the response was recorded as premature and the mouse was punished with a 10 s timeout. The stimulus duration was initially set to 4 s, followed by a limited holding period of 5 s, during which the stimulus was absent but the mouse could still respond to the location (holding period). Each session lasted 50 trials or 1 h. Responses to the stimulus window during stimulus presence or the holding period were recorded as correct, whereas responses to any other window were recorded as incorrect. A correct choice was rewarded with a tone and food delivery. An incorrect response was punished with a 10 s timeout. A failure to respond to any window either during stimulus display or the holding period was recorded as an omission, and the mouse was punished with a 10 s timeout. Perseverative responses to the screen after premature, correct, and incorrect choices were also recorded. Our initial goal was to have the performance of a mouse reaching criterion at 4 s stimulus duration (80% accuracy, 20% omissions for 3 consecutive days) and reduce the stimulus duration to 2 s. However, ChAT–ChR2–EYFP BAC mice were not able to reach criterion at 4 s stimulus duration. Therefore, we used another training procedure, that is, the same cohort of mice was trained in a 16 s stimulus duration, and when they reached criterion, the stimulus duration was reduced to 8 s. After reaching criterion with the 8 s stimulus, the mice were tested 2 more days, and the mean measures of those additional 2 d were used to assess baseline performance.

After finishing training at 8 s stimulus duration, mice were probed for attentional deficits in the following probe trial schedule: each mouse was tested over two sessions at a given stimulus duration (4 and 2 s). Between each different stimulus duration, the mouse was returned to an 8 s stimulus duration for two baseline sessions. The order of the probe trial sessions was semi randomized using a Latin square method.

On all 5-CSRT task sessions, accuracy was defined as the total number of correct responses divided by the number of correct and incorrect responses (touches to a wrong window while the correct stimulus was still displayed). Rate of omissions were the proportion of omitted responses to total trials. Response latency was the time for the mouse to touch the correct stimulus from its onset. Reward collection latency was the time for the mouse to return to the reward tray once it had touched the correct stimulus.

A premature response was counted when the mouse touched one of the windows before stimulus onset. Finally, a perseverative response was any identical response that occurred after a correct, incorrect, or premature response.

2.3.16 Statistical analyses

Data are expressed as mean ± SEM. SigmaStat 3.5 software was used for statistical analysis. Comparison between two experimental groups was done by Student's t test or Mann–Whitney rank-sum test when the data did not follow a normal distribution. When several experimental groups or treatments were analyzed, two-way ANOVA or two-way repeated-measures (RM) ANOVA were used as required. When appropriate, a Tukey's HSD post hoc comparison test was used.

2.4 Results

2.4.1 Increased levels of VAChT in the ChAT–ChR2–EYFP BAC mice

Immunofluorescence analysis confirmed previous findings that ChR2–EYFP is highly expressed in different areas of the brain (Zhao et al., 2011), including the striatum and basal forebrain (Fig. 2.1A), as well as interpeduncular nucleus and brainstem motor nuclei (data not shown). qPCR assays showed that ChAT–ChR2–EYFP mice contain ~56 copies of the VAChT gene and 54 copies of the YFP gene (Fig. 2.1B), suggesting that close to 50 copies of the ChAT–BAC were inserted in the mouse genome. RT-qPCR data indicate that these additional copies of the VAChT gene are functional because VAChT mRNA was increased almost 20-fold in the striatum of ChAT–ChR2–EYFP mice when compared with controls; importantly, expression of ChAT, as expected, was not changed (Fig. 2.1C). This increased VAChT mRNA level is consistent with the elevated copy number of ChAT–ChR2–EYFP BAC. Expression of the VAChT protein is also increased (Fig. 2.1D) in this mouse line. In the hippocampus, there is a 550% increase in VAChT protein levels, whereas in the brainstem, VAChT levels are augmented by 350% when compared with control littermates (Fig. 2.1D). Expression of the ChAT protein was unaltered in both brain regions (Fig. 2.1E).

Importantly, increased expression of VAChT protein had functional consequences, because ChAT–ChR2–EYFP BAC mice presented threefold to fourfold increase in the release of newly synthesized [3H]ACh from hippocampal slices (Fig. 2.1F). Moreover, BAC transgenic expression of VAChT was able to rescue postnatal lethality attributable to VAChT elimination. We crossed ChAT–ChR2–EYFP with VAChTdel/wt [heterozygous VAChT knock-out (de Castro et al., 2009b)] mice and then intercrossed heterozygous littermates to obtain ChaT–ChR2–EYFP VAChTdel/del. Screening of the offspring was done by qPCR of the VAChT del allele (de Castro et al., 2009b). Our data show that ChAT–ChR2–EYFP VAChTdel/del mice are viable and survive to adulthood (Table 1).

Figure 2.1- Overexpression of VAChT in ChAT–ChR2–EYFP mice. A, YFP expression in the CNS of ChAT–ChR2–EYFP mice (n = 3). B, VAChT and YFP gene copy numbers determined by genomic qPCR (n = 5). C, VAChT and ChAT mRNA expression in the striatum of wild-type and transgenic mice (n = 6 for both genotypes). D, VAChT protein expression in the hippocampus and brainstem with representative immunoblots (n = 3 for both genotypes). E, ChAT protein expression in the hippocampus and brainstem with representative immunoblots (n = 3 for both genotypes). F, Release of newly synthesized ACh in hippocampal slices (n = 4 for both genotypes). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 2.1- Overexpression of VAChT in ChAT–ChR2–EYFP mice.

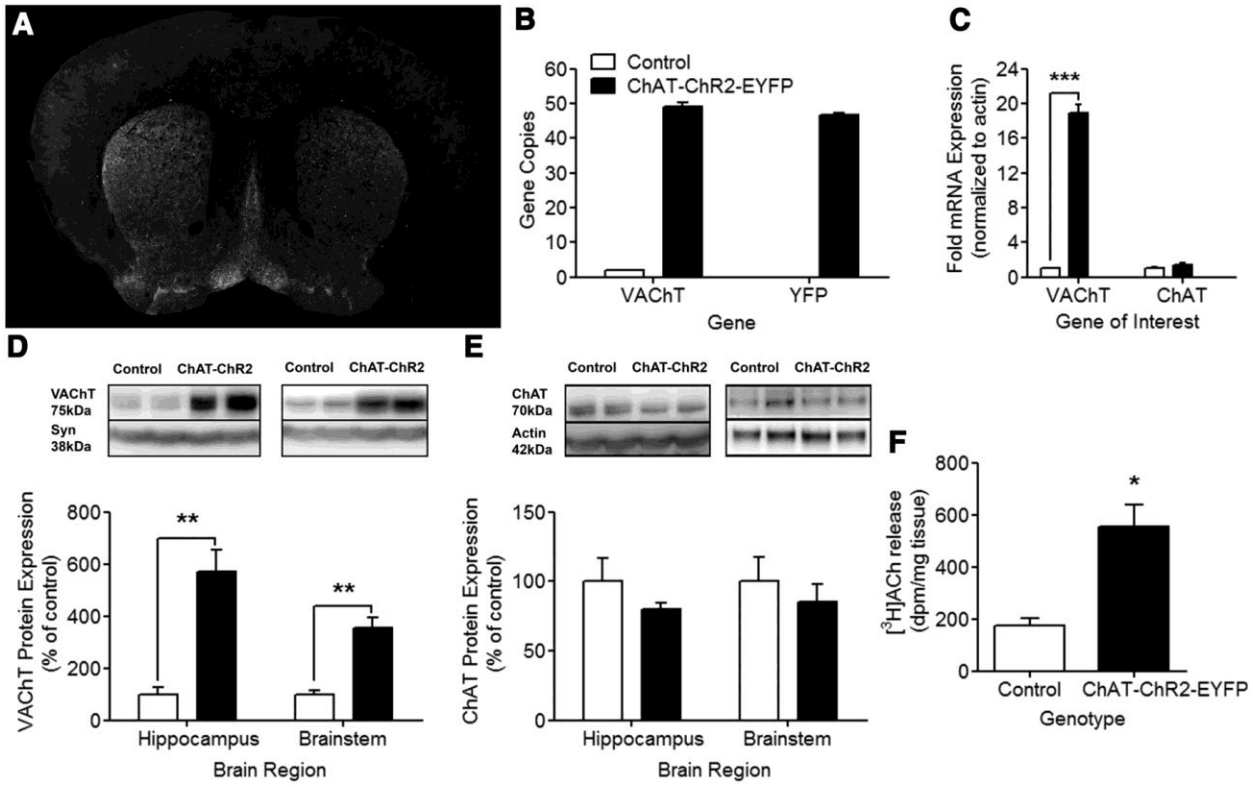


Table 2.1. Rescue of lethality in VAChTdel/del mice. ChAT–ChR2–EYFP mice were crossed to VAChTdel/wt mice and offspring were then intercrossed. Genomic qPCR using primers that amplify the VAChTdel allele was used to identify live VAChTdel/del mice containing the ChaT BAC.

Table 2.1. Rescue of lethality in VAChTdel/del mice.

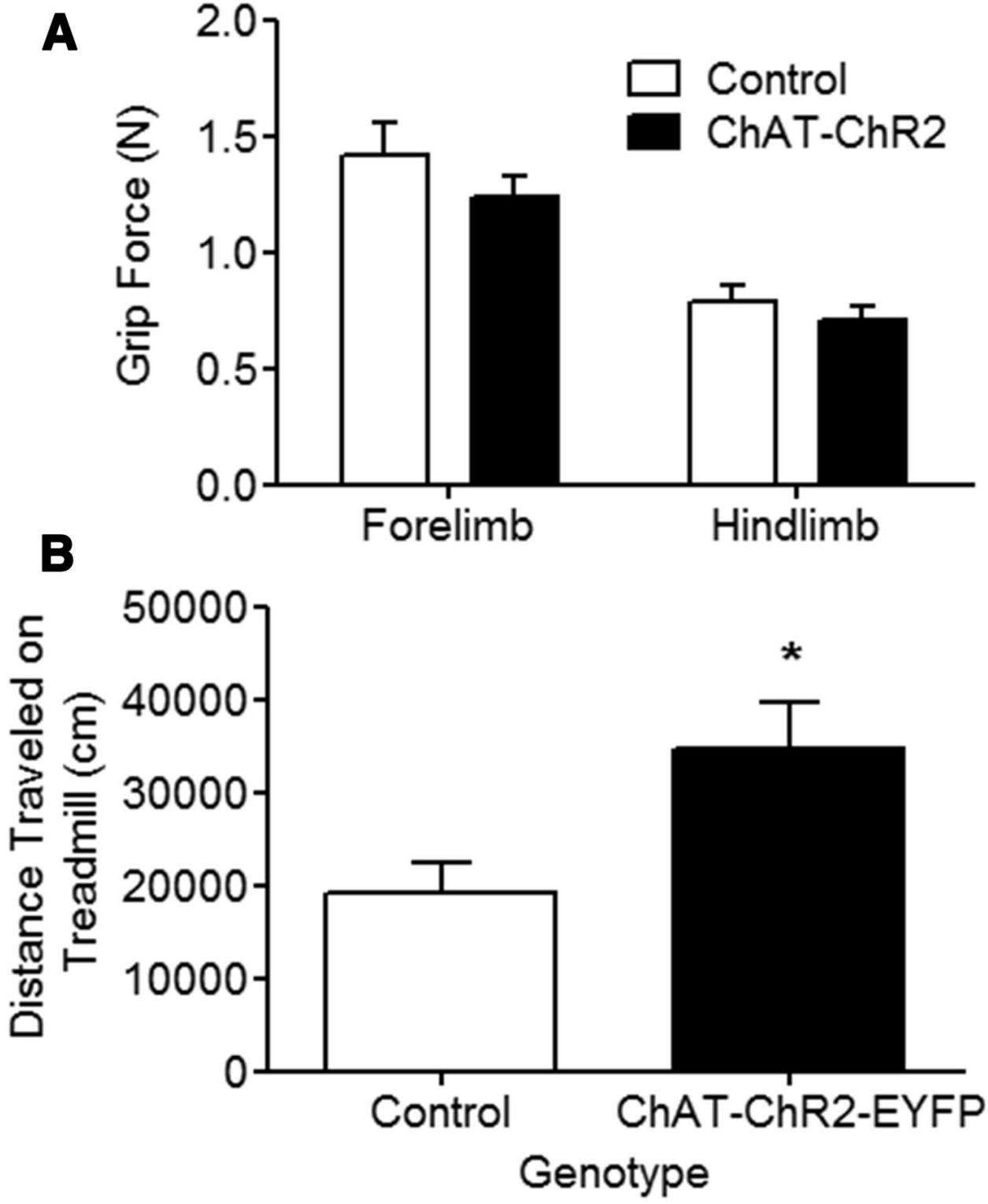
Genotype	Number of mice born
VAChT$^{wt/wt}$	2
VAChT$^{wt/del}$	7
ChAT–ChR2–EYFP VAChT$^{wt/del}$	10
ChAT–ChR2–EYFP VAChT$^{del/del}$	4
ChAT–ChR2–EYFP VAChT$^{wt/wt}$	6
Total	29

2.4.2 ChAT–ChR2–EYFP mice have improved motor endurance

To assess neuromuscular function in ChAT–ChR2–EYFP mice, both forelimb and hindlimb grip strength were measured, but there was no statistical difference between ChAT–ChR2–EYFP mice and control littermates (Fig. 2.2A). In contrast, ChAT–ChR2–EYFP mice performed much better than wild-type controls in the treadmill. By using a protocol designed to determine physical fitness, we observed that ChAT–ChR2–EYFP mice were able to run almost twice as much compared with control mice ($t(14) = 2.497$, $p = 0.0256$; Fig. 2.2B).

Figure 2.2 ChAT–ChR2–EYFP mice have increased physical endurance. A, Grip force in wild-type and transgenic mice. B, Treadmill analysis of physical endurance in wild-type and transgenic mice (*$p < 0.05$, $n = 8$ for both genotypes).

Figure 2.2 ChAT–ChR2–EYFP mice have increased physical endurance.

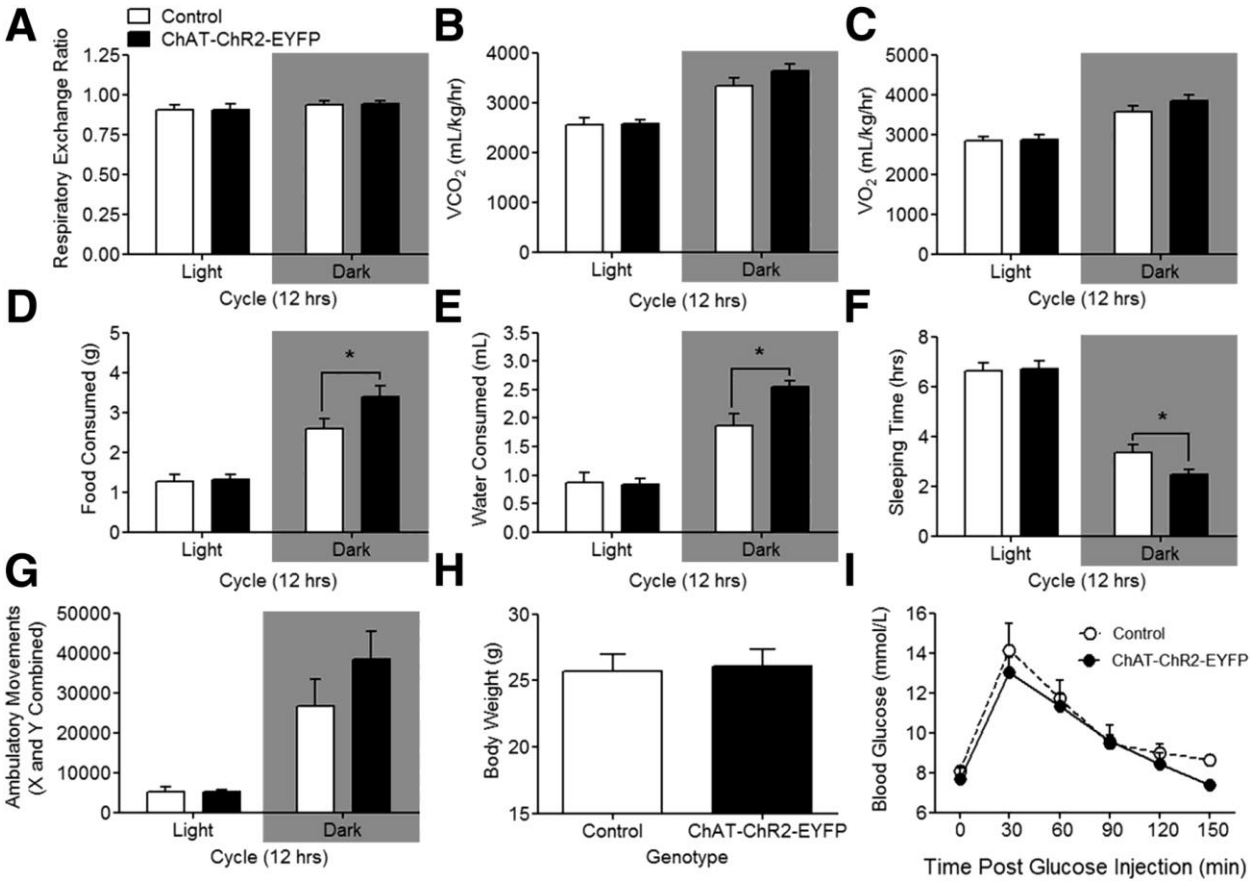


2.4.3 ChAT–ChR2–EYFP mice do not present gross alterations in metabolism

To investigate whether increased copy numbers of VAChT affects homeostasis, transgenic ChAT–ChR2–EYFP mice were assessed in metabolic cages. Transgenic mice had body weight statistically similar to controls ($t(14) = 0.6920$, $p = 0.500$; Fig. 2.3). These mice presented similar metabolic profiles as controls, with no statistical differences in RER, in the light ($t(14) = 0.9898$, $p = 0.831$) or the dark ($t(14) = 0.5414$, $p = 0.702$) cycles (Fig. 2.3A). They did not consume more O_2 during the light cycle ($t(14) = 0.1897$, $p = 0.8523$) or dark cycle ($t(14) = 1.402$, $p = 0.1828$; Fig. 2.3B). Similar results were obtained for CO_2 release during the light ($t(14) = 0.09952$, $p = 0.9221$) or dark ($t(14) = 1.462$, $p = 0.1658$) cycle (Fig. 2.3C). Likewise, locomotor activity, sleep time, or blood glucose levels were not significantly altered in this transgenic mouse line (Fig. 2.3). Interestingly, ChAT–ChR2–EYFP mice consumed both more food and water during the dark cycle than control mice (food consumption, $t(14) = 2.212$, $p = 0.0441$; water consumption, $t(14) = 2.878$, $p = 0.0122$; Fig. 2.3D,E).

Figure 2.3. Metabolic analysis in ChAT–ChR2–EYFP mice. A, Respiratory exchange rate analysis. B, VCO2 consumption. C, VO2. D, Food consumption. E, Water consumption. F, Sleep time. G, Home cage activity. H, Body weight at time of analysis. I, Glucose tolerance test. *$p < 0.05$, $n = 8$ for both genotypes.

Figure 2.3. Metabolic analysis in ChAT–ChR2–EYFP mice.

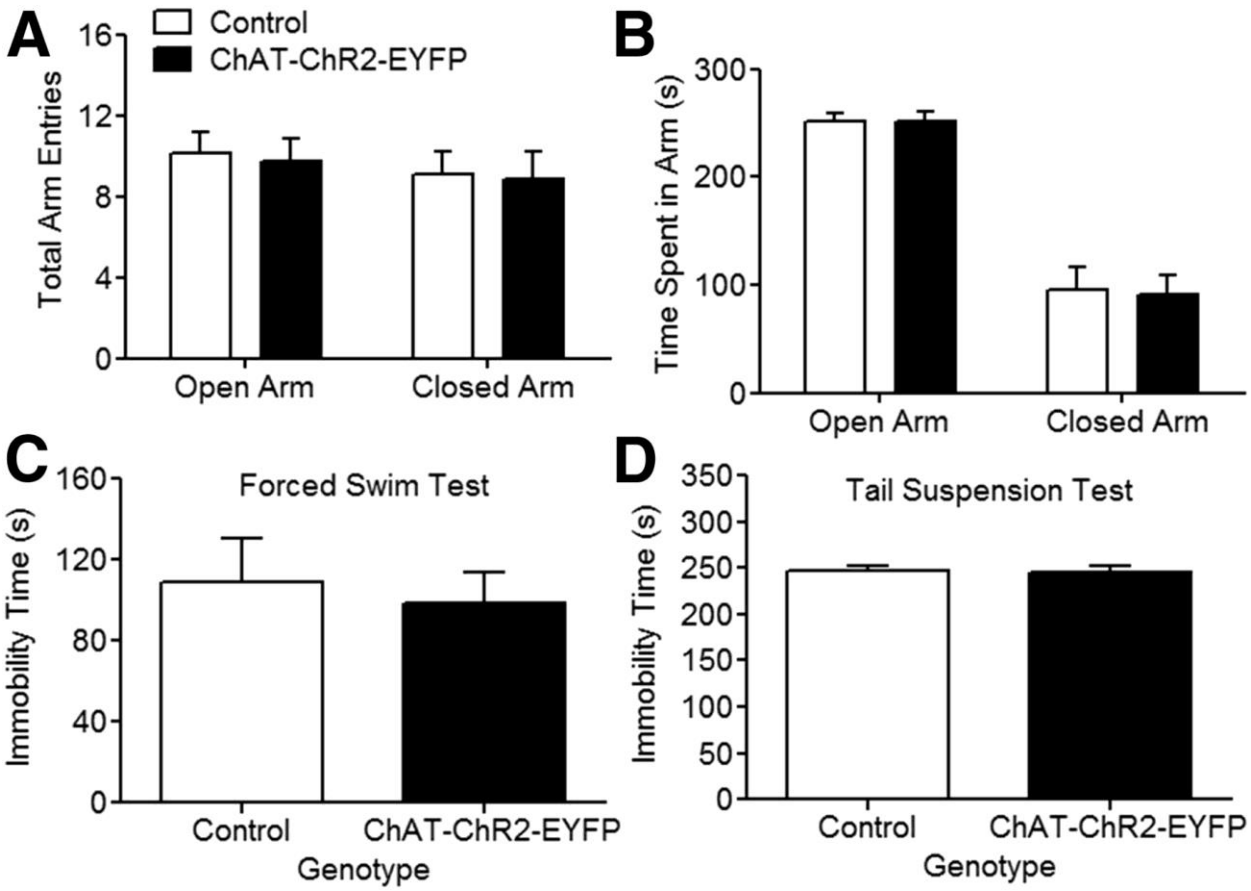


2.4.4 ChAT–ChR2–EYFP mice do not present anxiety or depression-like behavior

We tested ChAT–ChR2–EYFP mice for anxiety using the elevated plus maze paradigm. These mice visited the open ($t(14) = 0.2304$, $p = 0.8211$) or closed ($t(14) = 0.1365$, $p = 0.8934$) arms at rates statistically comparable with those observed in controls (Fig. 2.4A). In addition, they did not spend more time than controls in the open ($t(14) = 0.2304$, $p = 0.8211$) or closed ($t(14) = 0.1314$, $p = 0.8973$) arms (Fig. 2.4B). ChAT–ChR2–EYFP mice were also tested for depressive-like behavior using both the tail suspension and forced swim tests and presented no statistical difference from wild-type controls (swim test, $t(14) = 0.4016$, $p = 0.6941$; tail suspension, $t(14) = 0.04468$, $p = 0.9650$; Fig. 2.3C,D, respectively).

Figure 2.4. Assessment of anxiety and depressive-like behavior in ChAT–ChR2–EYFP mice. A, Number of arm entries in the elevated plus maze. B, Time spent in the open and closed arms. C, Immobility time in the forced swimming test. D, Immobility time in the tail suspension test (n = 8 for both genotypes).

Figure 2.4. Assessment of anxiety and depressive-like behavior in ChAT–ChR2–EYFP mice.



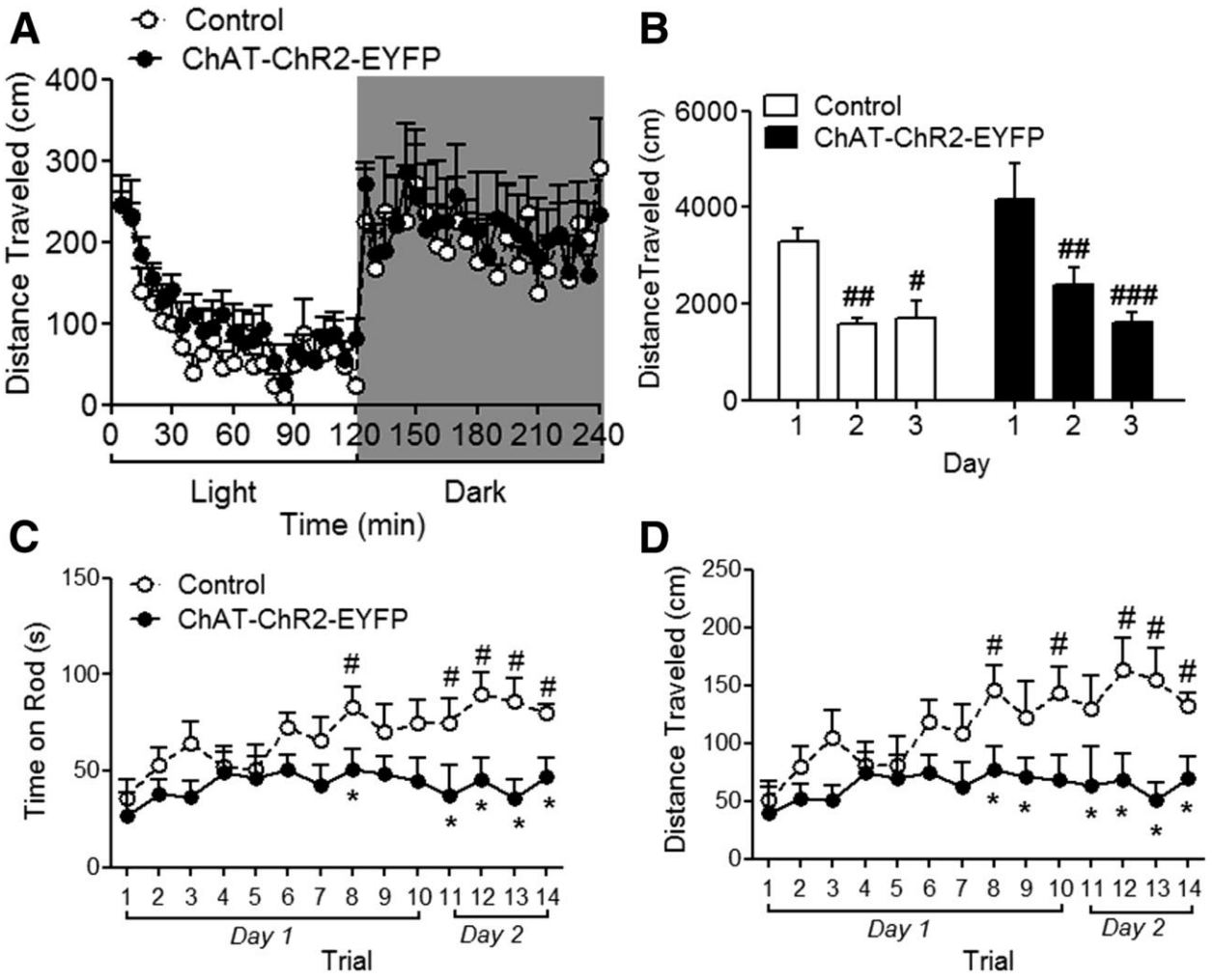
2.4.5 ChAT–ChR2–EYFP mice show normal locomotion but have impaired motor learning

We assessed locomotor activity in ChAT–ChR2–EYFP mice using an automated novel open-field environment for 4 h: 2 h in the light and 2 h in the dark. No statistical differences in locomotor activity were observed between genotypes ($F(1,658) = 0.2468$, $p = 0.6271$; Fig. 2.5A). There was no interaction between time × genotype ($F(47,658) = 0.4313$, $p = 0.9997$), with both genotypes significantly reducing their locomotor activity during the course of the test in the light phase ($F(47,658) = 9.725$, $p < 0.0001$). Moreover, habituation in the open field was not affected in ChAT–ChR2–EYFP mice [Fig. 2.5B; two-way RM-ANOVA shows a significant effect of day ($F(2,42) = 15.07$, $p < 0.0001$), no effect of genotype ($F(1,42) = 2.653$, $p = 0.1108$), and no interaction ($F(2,42) = 0.880$, $p = 0.4225$)].

Knowing that transgenic mice have increased endurance and no overt deficits in locomotor behavior, the accelerating rotarod task was used to assess motor learning. Surprisingly, the ChAT–ChR2–EYFP mice failed to improve their performance in the rotarod, whereas wild-type control mice improved the time spent on the rotarod as well as distance traveled [Fig. 2.5C,D; two-way RM-ANOVA revealed main effect of genotype ($F(1,182) = 6.015$, $p = 0.0279$) and trial ($F(13,182) = 2.796$, $p = 0.0012$)]. Post hoc analysis confirmed that the ChAT–ChR2–EYFP mice did not significantly improve their performance from their first trial.

Figure 2.5. ChAT–ChR2–EYFP mice have normal locomotor activity but impaired motor learning. A, Locomotor activity of wild-type or ChAT–ChR2–EYFP mice during the light (2 h) and dark (2 h) activity periods in an open arm. The test was done from 5:00 P.M. to 9:00 P.M. B, Habituation in the open field. C, Acrobatic motor performance and motor learning in the rotarod. Time spent on the rod. D, Distance traveled in the rotarod (*$p < 0.05$, significant difference between genotypes; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, significant difference within genotype; $n = 8$ for both genotypes).

Figure 2.5. ChAT–ChR2–EYFP mice have normal locomotor activity but impaired motor learning.



2.4.6 ChAT–ChR2–EYFP mice have impaired spatial memory

To assess other forms of learning, we tested spatial learning and memory in the ChAT–ChR2–EYFP mice using the Barnes maze and MWM. During the acquisition phase of the Barnes maze, there were no statistical differences for the performance between the two genotypes. The two groups made a similar number of errors before reaching the target hole ($F(1,42) = 0.2685$, $p = 0.6124$; Fig. 2.6A). In contrast, during the probe trial, on day 5, ChAT–ChR2–EYFP mice visited the target hole location significantly less than controls ($t(14) = 3.360$, $p = 0.0047$; Fig. 2.6B) and showed a significantly decreased preference for the target hole, as defined by the target hole preference index [target hole visits/mean visits per hole (Holmes et al., 2002), $t(14) = 2.712$, $p = 0.0168$; Fig. 2.6C]. Although both genotypes showed a significant effect of the hole location during the probe trial ($F(1,154) = 26.66$, $p = 0.001$), there was a significant effect of genotype ($F(1,154) = 11.29$, $p = 0.047$) and a significant genotype × hole location effect ($F(1,154) = 2.819$, $p = 0.022$). Post hoc analysis revealed that wild-type mice preferred the target hole, whereas ChAT–ChR2–EYFP mice did not (Fig. 2.6D,E). Representative traces of two controls (Fig. 2.6F) and two ChAT–ChR2–EYFP mice (Fig. 2.6G) show the performance of the two genotypes in this task.

To further determine the mechanisms involved with potential spatial memory deficits, the MWM was used. Once again during the course of acquisition, the performance of ChAT–ChR2–EYFP mice was indistinguishable from that of controls in terms of latency to find the target ($F(1,42) = 0.8933$, $p = 0.3606$) and the distance traveled to the target ($F(1,42) = 2.783$, $p = 0.1175$; Fig. 2.7A–C). Mutants learned the location of the platform similar to control mice, confirming the observations using the Barnes maze. These results indicate that the ChAT–ChR2–EYFP mice do not have any gross sensorimotor deficits and were able to use the cues to learn the task. However, on the probe trial day, ChAT–ChR2–EYFP mice showed no preference for the target quadrant of the pool (Fig. 2.7D). The occupancy plots in the MWM on the probe trial show that the controls clearly remembered where the platform should be (Fig. 2.7E). In contrast, ChAT–ChR2–EYFP mice did not seem to retrieve this information during the probe trial (Fig. 2.7F).

Figure 2.6. ChAT–ChR2–EYFP mice have spatial memory deficits in the Barnes maze. Mice were subject to the Barnes maze paradigm, and the average values of four 3-min trials per day are plotted. A, Number of errors before finding the target hole. B, Visits to the target hole during the day 5, 90 s probe trial. C, Preference for the target hole over other holes during the probe trial (target hole visits/average nontarget visits). D, Nose pokes per hole were measured on day 5 in a 90 s probe trial for control mice. T, Target hole. Numbers refer to the location of holes adjacent to the target hole. Op, Opposite hole. E, ChAT–ChR2–EYFP mice. F, Representative path tracings for two control mice during the probe trial (target quadrant highlighted; T). G, Same as in F but for ChAT–ChR2–EYFP mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 8$ for both genotypes.

Figure 2.6. ChAT–ChR2–EYFP mice have spatial memory deficits in the Barnes maze.

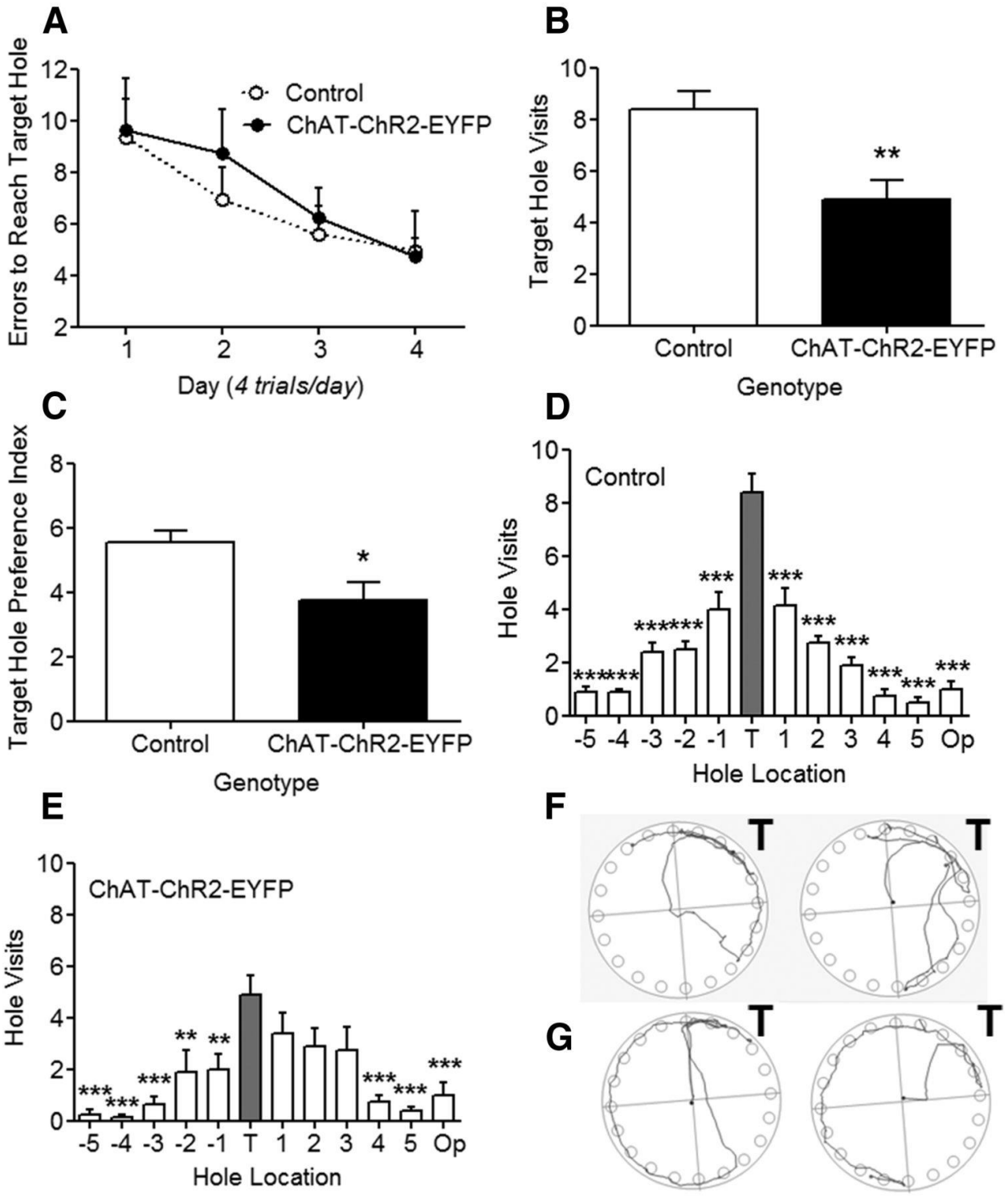
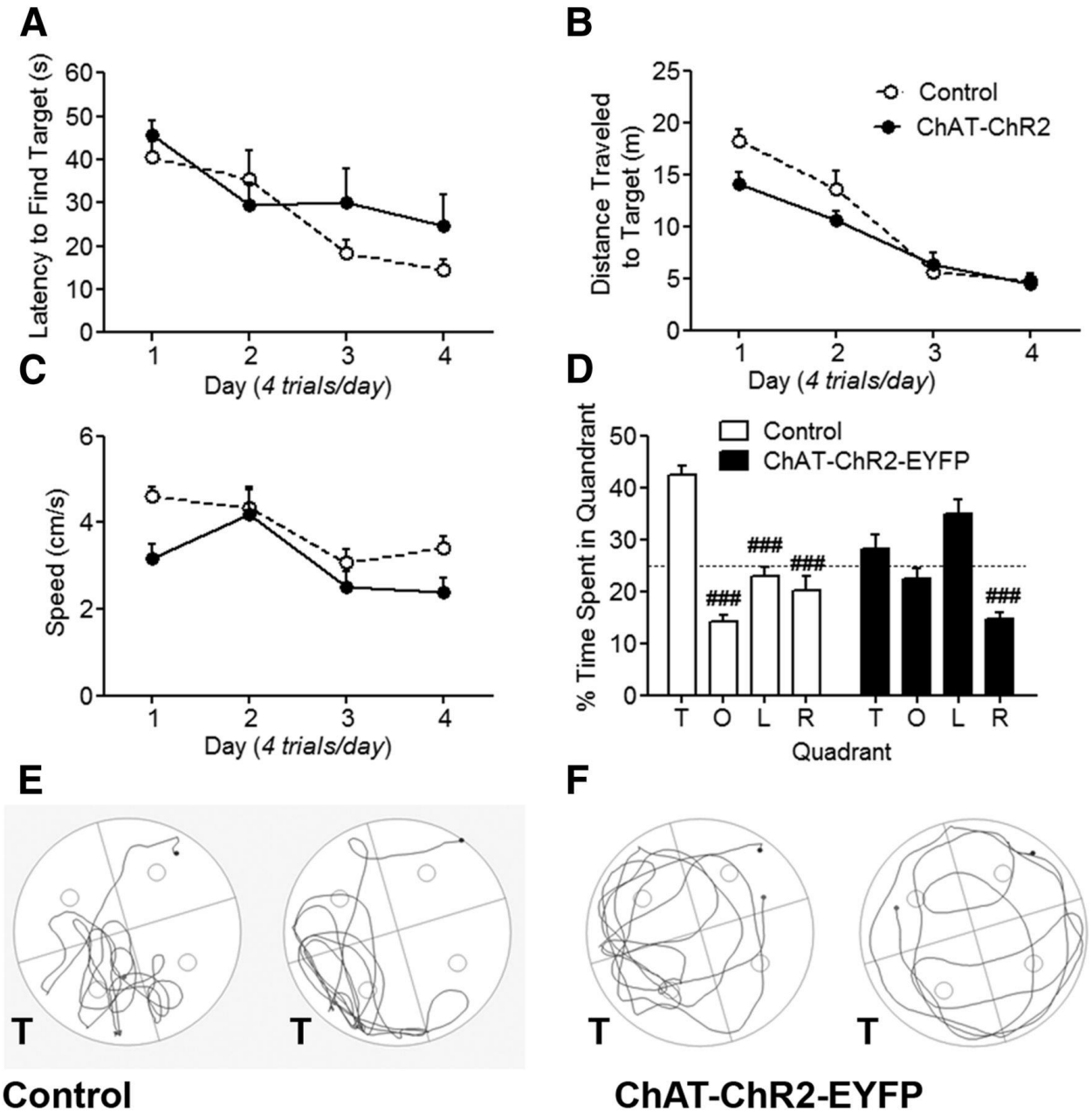


Figure 2.7. ChAT–ChR2–EYFP mice have spatial memory deficits in the MWM. Mice were subject to the MWM paradigm, and the average values of four 90-s trials per day are plotted. A, Latency to find the platform. B, Distance traveled to the platform. C, Mouse speed. D, Percentage time spent per quadrant was measured on day 5 in a 60 s probe trial with the platform removed. E, Representative occupancy plots for two control mice. F, Representative occupancy plots for two ChAT–ChR2–EYFP mice during the probe trial (T indicates the location of the target quadrant). ###$p < 0.001$, significant difference within genotype, $n = 8$ for both genotypes. L, Left; O, opposite; R, right; T, target.

Figure 2.7. ChAT–ChR2–EYFP mice have spatial memory deficits in the MWM.

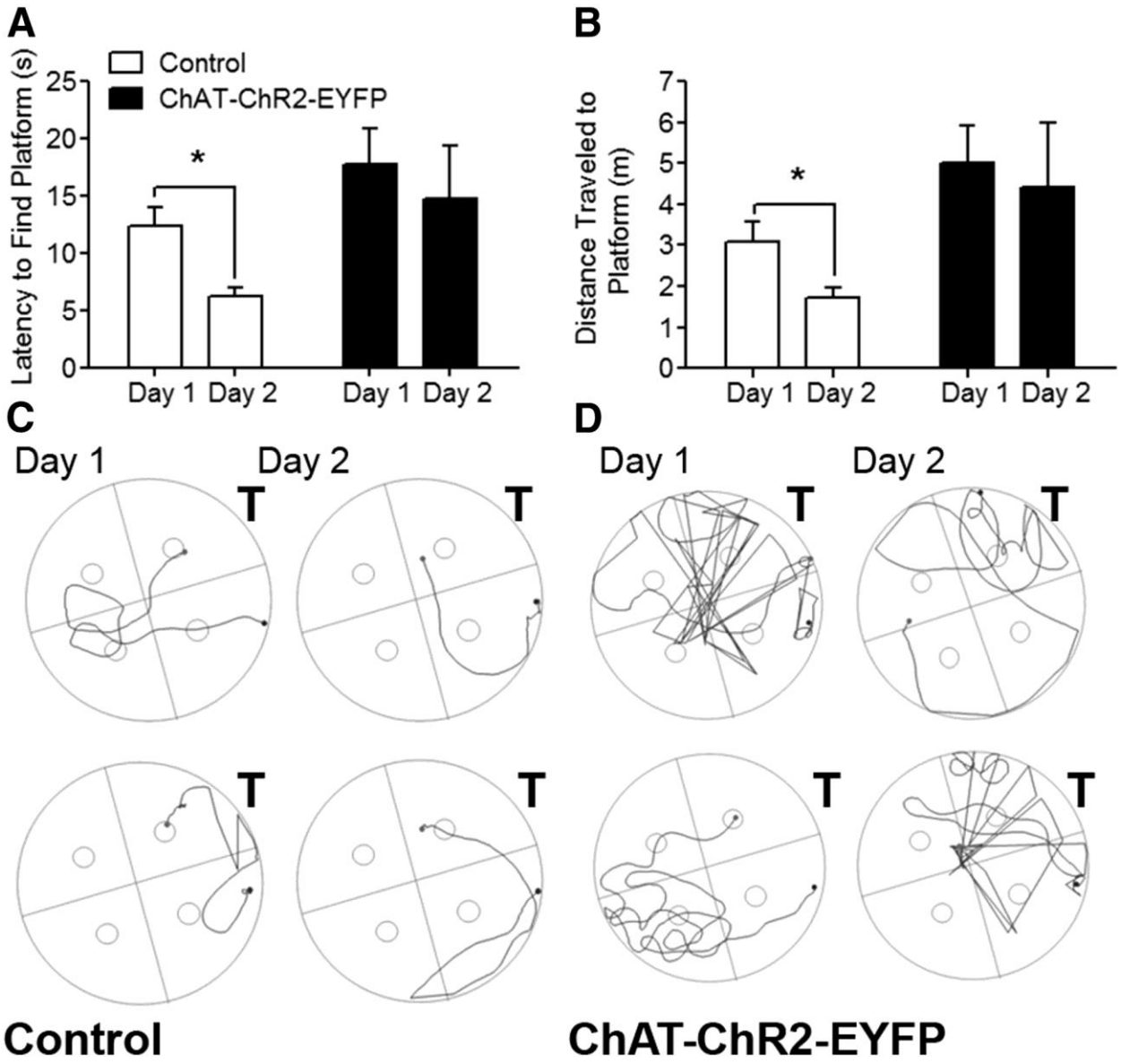


2.4.7 ChAT–ChR2–EYFP mice have deficiencies in cue-directed memory

To assess cue-driven learning in these mice, the cued version of the MWM was used (Vorhees and Williams, 2006). There was a significant difference between genotypes ($F(1,14) = 5.262$, $p = 0.0378$) and a significant effect of day ($F(1,14) = 7.834$, $p = 0.0142$) in terms of latency to find the target (Fig. 2.8A). Post hoc analysis confirmed that control mice improved their performance from day 1 to day 2, whereas ChAT–ChR2–EYFP mice did not (Fig. 2.8A). There was a trend for ChAT–ChR2–EYFP mice to swim a greater distance to the target (Fig. 2.8B), but this failed to reach significance ($F(1,14) = 7.834$, $p = 0.0861$). A closer examination of the path traces revealed that ChAT–ChR2–EYFP mice do not seem to use the cue to find the target on the second day (Fig. 2.8C,D).

Figure 2.8. ChAT–ChR2–EYFP mice have deficits in cue memory. Mice were subject to the cued version of the MWM in which they had to associate the platform with a cue. A, Primary latency to find the platform. B, Distance traveled to the platform. C, Representative path tracings to the target of two control mice. D, Representative path tracings to the target of two ChAT–ChR2–EYFP mice. Traces are from the second trial on the first and second days of the experiment, with T indicating the location of the platform. The average of four 90-s trials per day is plotted. *$p < 0.05$, $n = 8$ for both genotypes.

Figure 2.8. ChAT–ChR2–EYFP mice have deficits in cue memory.



2.4.8 ChAT–ChR2–EYFP have impaired working memory

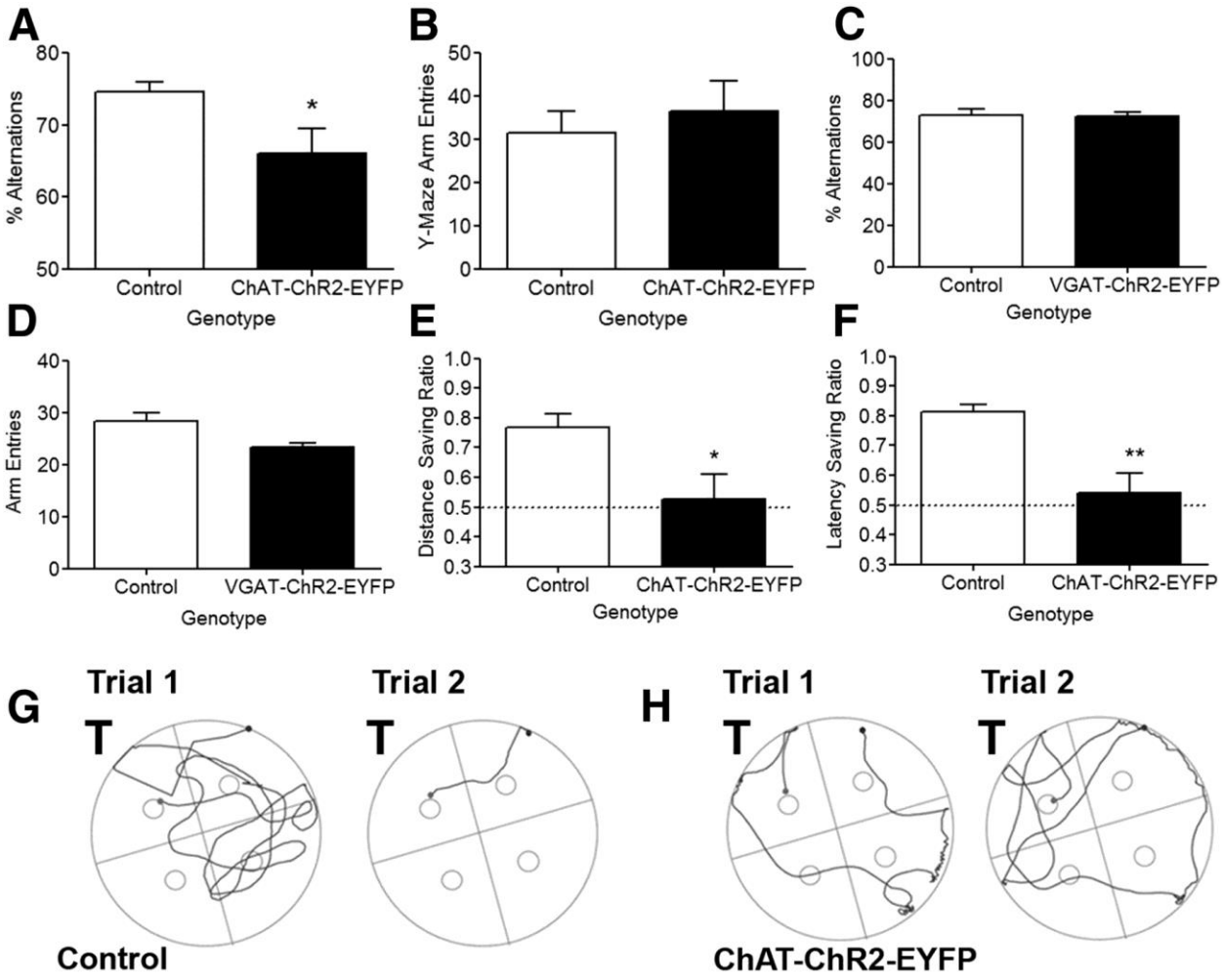
The above experiments suggest that ChAT–ChR2–EYFP mice can learn the spatial version of the MWM, but they have difficulty retrieving that memory trace. Moreover, these transgenic mice show impaired motor learning and cued-driven learning. These results suggest the possibility that chronically increased cholinergic tone disturbs distinct forms of information processing. To evaluate working memory, we first used spontaneous alternations in the Y-maze (de Castro et al., 2009a). In contrast to results obtained with littermate control mice, ChAT–ChR2–EYFP mice revisited the arms of the Y-maze more often, showing significantly less spontaneous alternations ($t(14) = 2.448$, $p = 0.0293$; Fig. 2.9A). The number of arm entries was not affected ($t(14) = 0.6031$, $p = 0.5568$; Fig. 2.9B), nor was distance traveled ($t(14) = 0.5620$, $p = 0.5837$; data not shown). However, performance of ChAT–ChR2–EYFP mice was above chance (>50%) regarding alternations, suggesting that these mice had a partial dysfunction on their working memory. To exclude the possibility that the cognitive deficits observed could be related to the introduction of ChR2 or EYFP, we also tested VGAT–ChR2–EYFP and their littermate controls in the Y-maze alternation. qPCR analysis showed that these mice had ~20 copies of EYFP and therefore 20 copies of the ChR2 gene. Distinct from ChAT–ChR2–EYFP mice, VGAT–ChR2–EYFP mice did not differ statistically from their littermate controls in terms of either spontaneous alternations ($t(10) = 0.1914$, $p = 0.8520$; Fig. 2.9C) or number of arm entries ($t(10) = 1.562$, $p = 0.1494$; Fig. 2.9D).

To further probe working memory in ChAT–ChR2–EYFP mice, the two-trial variation of the MWM was used (Vorhees and Williams, 2006). In this variation of the task, mice must first find a novel platform location by chance and then, after a 10 s ITI, find it again. To analyze mouse performance on this task, we used the latency and distance savings ratio to standardize data (Varvel and Lichtman, 2002). These ratios were calculated by dividing the distance traveled, or latency on the first trial by that on the sum of the first and second trials. Values >0.5 indicate improvement from the first to the second trial. ChAT–ChR2–EYFP mice had significantly lower distance savings ratio ($t(14) = 2.501$, $p = 0.0254$) and latency savings ratio ($t(14) = 3.684$, $p = 0.0025$; Fig.

2.9E–H) than control mice. Together, these results indicate that ChAT–ChR2–EYFP mice have impaired working memory.

Figure 2.9. ChAT–ChR2–EYFP mice have deficits in working memory. A, Spontaneous alternations in the Y-maze were used to assess working memory for ChAT–ChR2–EYFP mice. B, Number of arms visited for ChAT–ChR2–EYFP mice (n = 8 for both genotypes). C, Spontaneous alternations in the Y-maze for VGAT–ChR2–EYFP mice. D, Number of arms visited for VGAT–ChR2–EYFP mice. n = 6 for both genotypes. E, ChAT–ChR2–EYFP mice were subject to the two-trial MWM paradigm to further assess working memory. Savings ratios were calculated from the average of the first and second 90 s trials across all 4 d of the experiment. The graph shows the mean distance savings ratios. F, Latency saving ratio. G, Representative path tracings to the target for two control mice. H, Representative path tracings to the target for two ChAT–ChR2–EYFP mice. Traces are from the first and second trials during the third day of the experiment. T indicates the location of the platform. *$p < 0.05$, **$p < 0.01$, n = 8 for both genotypes.

Figure 2.9. ChAT–ChR2–EYFP mice have deficits in working memory.



2.4.9 ChAT–ChR2–EYFP mice have impaired attentional processing

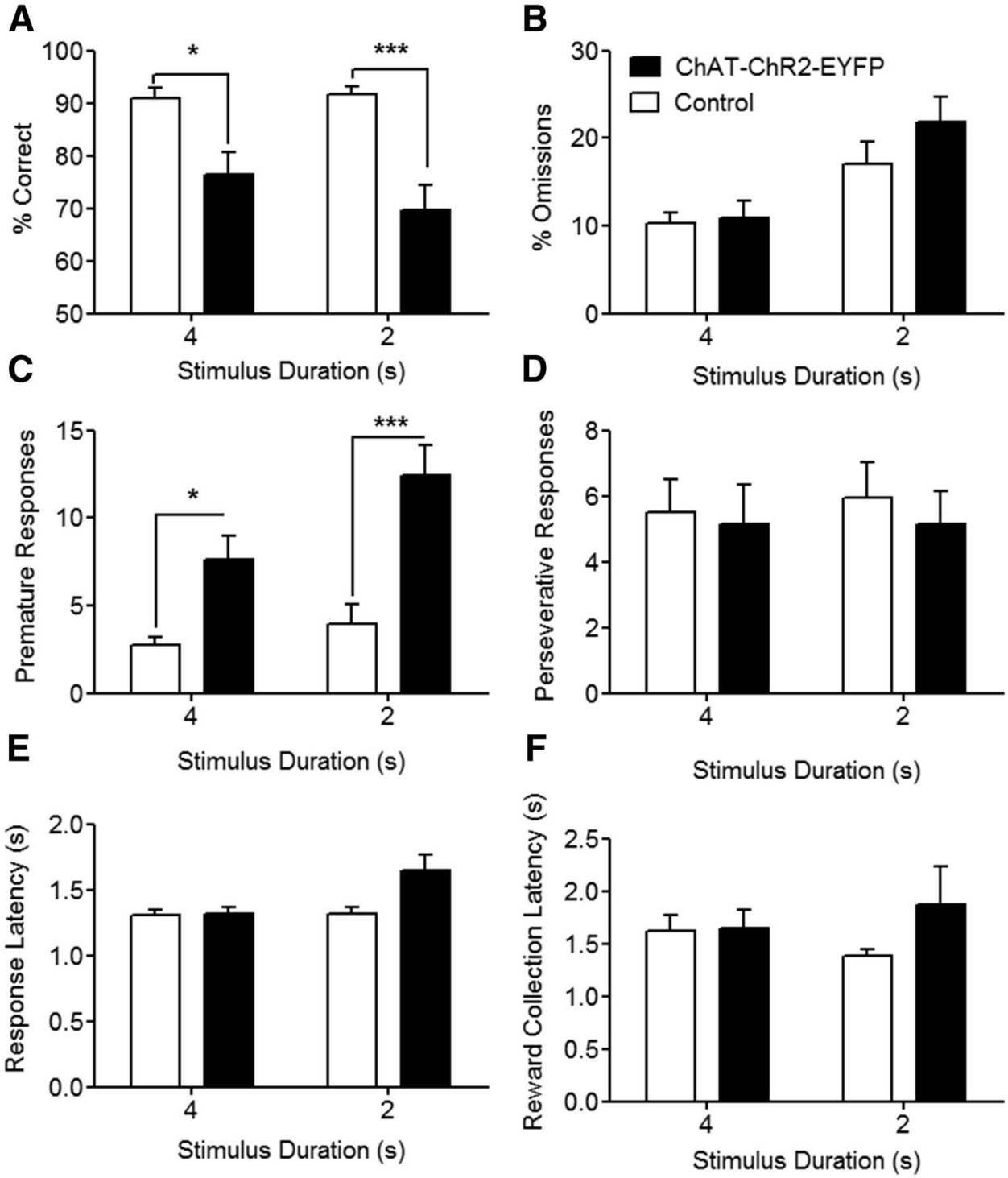
To determine whether ChAT–ChR2–EYFP mice may be affected in other cognitive domains that are sensitive to cholinergic tone and could contribute to the deficits we observed, we used the 5-CSRT task. During pretraining, transgenic mice did not differ from controls in terms of trials needed to achieve criterion [two-way RM-ANOVA; no effect of genotype ($F(1,48) = 0.6766$, $p = 0.4268$) and no effect of training phase ($F(4,48) = 2.306$, $p = 0.0717$)]. It should be noted that one mouse of each genotype never completed the pretraining and were not subjected to training in the 5-CSRT task. Our initial goal was to train mice first using a 4 s stimulus duration and then proceed to 2 s as described previously (Romberg et al., 2011). However, after 12 training sessions at 4 s stimulus duration, whereas all control mice reached criterion with an average of 10.28 ± 1.23 trials, all ChAT–ChR2–EYFP mice failed to acquire the task. Therefore, we increased the stimulus duration time (decreased the attentional demand) to 16 s and then 8 s to do the training. Probe trials were then performed with 4 and 2 s stimulus durations.

Under less demanding attentional conditions, ChAT–ChR2–EYFP mice were able to acquire the task, reaching criteria at both stimulus durations (16 and 8 s) in the same number of sessions as controls [two-way RM-ANOVA; no effect of genotype ($F(1,12) = 1.122$, $p = 0.3103$) and no effect of stimulus duration ($F(1,12) = 2.492$, $p = 0.1404$)]. However, during probe trials, ChAT–ChR2–EYFP mice showed significant impairments in choice accuracy [two-way RM-ANOVA; main effect of genotype ($F(1,12) = 29.86$, $p = 0.0001$) and no effect of stimulus duration ($F(1,12) = 0.6894$, $p = 0.4226$); Fig. 2.10A], with post hoc analysis showing that the ChAT–ChR2–EYFP mice had impaired accuracy at both stimulus durations. The rate of omissions for ChAT–ChR2–EYFP mice was unaffected [two-way RM-ANOVA; no effect of genotype ($F(1,12) = 1.928$, $p = 0.1902$) and main effect of stimulus duration ($F(1,12) = 13.79$, $p = 0.0030$); Fig. 2.10B]. Additionally, ChAT–ChR2–EYFP mice showed a significant increase in premature

responses [two-way RM-ANOVA; main effect of genotype ($F(1,12) = 21.74$, $p = 0.0005$) and main effect of stimulus duration ($F(1,12) = 7.657$, $p = 0.0171$); Fig. 2.10C]. Post hoc analysis revealed that the ChAT–ChR2–EYFP mice had more premature responses at each stimulus duration. No change in perseverative responses was observed in ChAT–ChR2–EYFP mice [two-way RM-ANOVA; no effect of genotype ($F(1,12) = 0.05002$, $p = 0.8268$) and no effect of stimulus duration ($F(1,12) = 0.6894$, $p = 0.4226$); Fig. 2.10D]. Importantly, there were no differences between genotypes in terms of response latency [two-way RM-ANOVA; no effect of genotype ($F(1,12) = 1.570$, $p = 0.2341$) and no effect of stimulus duration ($F(1,12) = 2.112$, $p = 0.1718$)] or reward collection latency [two-way RM-ANOVA; no effect of genotype ($F(1,12) = 1.082$, $p = 0.3189$) and no effect of stimulus duration ($F(1,12) = 0.001394$, $p = 0.9708$); Fig. 2.10E,F].

Figure 2.10. ChAT–ChR2–EYFP mice have deficits in attention. The 5-CSRT task was used to measure attention in the ChAT–ChR2–EYFP mice. A, Mean response accuracy during probe trial sessions. B, Rate of omissions. To assess response patterns, both premature (C) and perseverative (D) responses were monitored during probe trials. E, Mean response latency. F, Mean reward collection latency. *$p < 0.05$, ***$p < 0.001$, $n = 7$ for both genotypes.

Figure 10. ChAT–ChR2–EYFP mice have deficits in attention



2.5 Discussion

Here we report an extensive characterization of the BAC transgenic ChAT–ChR2–EYFP mouse line and reveal two major findings. First, there are several extra copies of the VAChT gene in this mouse line, which led to increased levels of functional transporter, increased release of ACh, and improvement of physical endurance. Second, it seems that this chronic increase in cholinergic tone is deleterious in the CNS, disrupting several distinct cognitive domains. Interestingly, increase in VAChT mRNA expression was approximately fourfold higher than increase in protein expression. This result suggests the existence of translational or posttranslational mechanisms limiting the availability of the VAChT protein in cholinergic neurons.

Vesicular storage of ACh is a required step for ACh release (de Castro et al., 2009b), and decreased VAChT expression leads to motor and cognitive dysfunctions (Prado et al., 2006; Martyn et al., 2012). Specifically, reduction of VAChT levels affects grip strength and fatigue; VAChT knockdown homozygous mice, with 70% reduction in VAChT levels, are unable to run in a treadmill test (Prado et al., 2006). In agreement with these early results, overexpression of the high-affinity choline transporter in motoneurons improves the performance of transgenic mice in the treadmill (Lund et al., 2010). The present results with ChAT–ChR2–EYFP mice further support the notion that physical fitness is related to changes in cholinergic synaptic activity. Increased VAChT levels allowed transgenic mice to run farther than control nontransgenic littermates on the treadmill. However, it is unknown whether the increase in motor endurance results only from increased VAChT levels in motoneurons because, in this mouse line, VAChT is likely to be overexpressed in all cholinergic nerve endings. Decreased VAChT expression in the periphery has been shown to affect cardiac activity by distinct mechanisms (Lara et al., 2010; Rocha-Resende et al., 2012; Roy et al., 2012; Prado et al., 2013). Thus, ChAT–ChR2–EYFP mice will be essential to determine whether increased VAChT expression can improve cardiovascular function.

Although a number of studies indicate that ACh is involved in the regulation of metabolic homeostasis (for review, see Picciotto et al., 2012), no significant difference between

ChAT–ChR2–EYFP and wild-type littermates was observed when an extensive list of metabolic parameters was analyzed, including RER, the volume of oxygen produced (VO2), the volume of carbon dioxide produced (VCO2), body weight, sleeping time, ambulatory movement, and serum glucose. However, our data show that ChAT–ChR2–EYFP mice consume significantly more food and water during the dark period. It is possible that the small increase in food and water intake observed in these transgenic mice reflects the fact that, in the home-cage-like environment, they have a small tendency to be more active than controls. Surprisingly, ChAT–ChR2–EYFP mice did not show changes in locomotor activity in a novel environment, anxiety-like behavior, or depression-like behavior. To note, increased cholinergic transmission has been suggested to be pro-depressive (Janowsky et al., 1972; Overstreet et al., 1986; Overstreet, 1993; Fagergren et al., 2005). Moreover, antagonists of nicotinic and muscarinic receptors have antidepressant activity (Rabenstein et al., 2006; Andreasen and Redrobe, 2009). Because chronic increase in cholinergic tone may affect the expression of distinct receptors in specific brain regions, it is unlikely that all phenotypes described previously attributable to cholinesterase inhibition or dysfunction may be affected in ChAT-ChR2-EYFP mice.

Despite their increased motor endurance, ChAT–ChR2–EYFP mice were unable to improve their performance in the rotarod, suggesting that increased cholinergic tone in the CNS is deleterious for learning an acrobatic skill. Previous experiments with mice presenting reduced levels of VAChT (40% VAChT knockdown heterozygous mice) had indicated that motor learning depends on cholinergic tone (Prado et al., 2006; de Castro et al., 2009b). Together, these results suggest that either too much or too little ACh in the brain is detrimental for motor learning.

We have also detected diminished performance of ChAT–ChR2–EYFP mice in two distinct tasks designed to measure spatial memory. In both the Barnes maze and MWM, ChAT–ChR2–EYFP mice present a specific deficit in the retrieval of information. In both tests, ChAT–ChR2–EYFP mice were able to learn the task (location of the platform or the exit hole), as evidenced by their improved performance over the 4 d of training. However, in probe trials, ChAT–ChR2–EYFP mice performed poorly compared with

their littermate controls in both tests. This phenotype may not be related only to spatial memory deficits, because these mice also presented impairments in the cued version of the MWM. Because ChAT–ChR2–EYFP mice can improve their performance during the 4 d of training in the Barnes maze and MWM, it is unlikely that they present any gross sensory motor deficits that would preclude visualization of cues. Conversely, deficits in the cued version of the MWM suggest that increased cholinergic tone might interfere with the mouse's ability to recognize that the platform is the goal (cue detection). It has been reported that detection of signals depends on cholinergic neurotransmission (Sarter et al., 2005; Parikh et al., 2007). Therefore, the chronic excess of cholinergic tone may disrupt the transmission of salient information related to a cue, preventing these animals from using such information to guide them to their goal.

Working memory is a prefrontal cortical process that is modulated by cholinergic signaling (Croxson et al., 2011). There is strong evidence implicating cholinergic activity in enhancing discrimination of signal-to-noise in the prefrontal cortex (for review, see Hasselmo and Sarter, 2011), a role that is critical in regulating attention (Sarter et al., 2006; Parikh et al., 2007; Hasselmo and Sarter, 2011). However, the focus in the literature has been on hypocholinergic function (McGaughy et al., 2002; Dalley et al., 2004; Harati et al., 2008; Parikh et al., 2013), and to date, there has not been an evaluation of chronic cholinergic deregulation on attentive processing. Additionally, ACh has been shown to be important for feature binding, the process by which the brain processes specific features of an object and compile a unified picture of it (Botly and De Rosa, 2009). Interestingly ChAT–ChR2–EYFP mice showed inattentive behavior related to cholinergic dysregulation; however, they also showed increased premature responses in the 5-CSRT task, a behavior that has been shown to be regulated by serotonergic signaling (Fletcher et al., 2013; Humpston et al., 2013). ACh has been proposed to help filter sensory information by increasing persistent spiking in cortical neurons, compatible with its proposed role in facilitating cue detection (Hasselmo and Stern, 2006). Such a mechanism depends on background tonic level of ACh but also on the transient increase in cholinergic activity (Parikh et al., 2007). Whether tonic cholinergic activity in the ChAT–ChR2–EYFP mice is so high that it precludes additional transient increases in cholinergic tone remains to be determined. However, the

observation that ChAT–ChR2–EYFP mice have working memory and attention deficits suggests the possibility that multiple forms of information encoding are affected in these mice. Therefore, these experiments emphasize the importance of regulated ACh release in cognitive function.

The precise mechanism by which excessive release of ACh in ChAT–ChR2–EYFP mice affects memory is not yet clear. It is likely that cellular regulation of neuronal spiking (Hasselmo and Sarter, 2011) may be affected by chronically increased levels of extracellular ACh. Moreover, increased and sustained cholinergic tone may affect forebrain circuitries by changing the expression of receptors and the regulation of other neurochemical systems, leading to abnormal processing, encoding, or retrieval of information. The overall memory deficits observed in ChAT–ChR2–EYFP mice suggest the need for specific temporal and spatial control of synaptic ACh levels for optimal cognitive performance.

In contrast with the worse performance of ChAT–ChR2–EYFP mice in cognitive tasks, augmented cholinergic tone seems to improve physical fitness, suggesting that increasing cholinergic tone may be beneficial in the periphery. Whether increased cholinergic function will be beneficial in other parameters regulated by the autonomic nervous system, as well as for improving the activity of the cholinergic anti-inflammatory pathway, remains to be determined.

Given that we detect improvement physical endurance and decreased performance in a series of behavioral tasks that have been previously related to cholinergic functions, we have interpreted our results as a potential consequence of increased cholinergic tone. It should be noted that we cannot eliminate the possibility that the large number of copies of the BAC inserted in the mouse genome disrupted a specific gene locus that could both improve physical endurance and disrupt cognition, although this seems unlikely. Also, we cannot discard the possibility that the high copy number of ChR2 or YFP may have unexpected consequences. We attempted to test this possibility by using another mouse line with high copy number of ChR2 and YFP. The VGAT–ChR2–EYFP mice, which have 20 copies of these genes, did not present impairments in working memory. These results support the argument that the effects observed in ChAT–ChR2–EYFP

mice are related to cholinergic hyperfunction. Importantly, these altered phenotypes fundamentally differentiate ChAT–ChR2–EYFP from control mice.

In short, our experiments indicate that ChAT–ChR2–EYFP mice overexpress VAChT and show important functional consequences, including unforeseen effects in cognitive processing. Because most studies using optogenetic control of cholinergic neurons have used mice expressing Cre or ChR2 that was inserted in the ChAT–BAC (Witten et al., 2010; Bell et al., 2011; Gu and Yakel, 2011; Nagode et al., 2011; Cachope et al., 2012; Kalmbach et al., 2012), it is important to be aware that VAChT overexpression may contribute to behavioral or cellular outputs. Therefore, novel approaches to control cholinergic neurons using optogenetics may be necessary. Inactivation of the VAChT gene in the ChAT–BAC is a possible alternative. However, ChAT–ChR2–EYFP mice will be valuable to test current theories of cholinergic function and the consequences of overactive cholinergic signaling for information processing.

2.6 Acknowledgements

This work was supported by Canadian Institutes of Health Research Grants MOP 89919 and 126000 (M.A.M.P. and V.F.P.), Natural Sciences and Engineering Research Council of Canada (V.F.P.), Heart and Stroke Foundation of Ontario Grant NA 6656 (M.A.M.P., V.F.P., and R.G.), Canada Foundation for Innovation (M.A.M.P., V.F.P., and R.G.), Ontario Research Fund (M.A.M.P., V.F.P., and R.G.), and a fellowship (M.S.G.) and a New Investigator Award (R.G.) from the Heart and Stroke Foundation of Canada.

2.7 References

Andreasen JT, Redrobe JP (2009) Antidepressant-like effects of nicotine and mecamylamine in the mouse forced swim and tail suspension tests: role of strain, test and sex. Behav Pharmacol 20:286–295.

Bejanin S, Cervini R, Mallet J, Berrard S (1994) A unique gene organization for two cholinergic markers, choline acetyltransferase and a putative vesicular transporter of acetylcholine. J Biol Chem 269:21944–21947.

Bell KA, Shim H, Chen CK, McQuiston AR (2011) Nicotinic excitatory postsynaptic potentials in hippocampal CA1 interneurons are predominantly mediated by nicotinic receptors that contain alpha4 and beta2 subunits. Neuropharmacology 61:1379–1388.

Botly LC, De Rosa E (2009) Cholinergic deafferentation of the neocortex using 192 IgG-saporin impairs feature binding in rats. J Neurosci 29:4120–4130.

Cachope R, Mateo Y, Mathur BN, Irving J, Wang HL, Morales M, Lovinger DM, Cheer JF (2012) Selective activation of cholinergic interneurons enhances accumbal phasic dopamine release: setting the tone for reward processing. Cell Rep 2:33–41.

Cervini R, Houhou L, Pradat PF, Béjanin S, Mallet J, Berrard S (1995) Specific vesicular acetylcholine transporter promoters lie within the first intron of the rat choline acetyltransferase gene. J Biol Chem 270:24654–24657.

Changeux JP (2010) Nicotine addiction and nicotinic receptors: lessons from genetically modified mice. Nat Rev Neurosci 11:389–401.

Croxson PL, Kyriazis DA, Baxter MG (2011) Cholinergic modulation of a specific memory function of prefrontal cortex. Nat Neurosci 14:1510–1512.

Dalley JW, Theobald DE, Bouger P, Chudasama Y, Cardinal RN, Robbins TW (2004) Cortical cholinergic function and deficits in visual attentional performance in rats following 192 IgG-saporin-induced lesions of the medial prefrontal cortex. Cereb Cortex 14:922–932.

de Castro BM, Pereira GS, Magalhães V, Rossato JI, De Jaeger X, Martins-Silva C, Leles B, Lima P, Gomez MV, Gainetdinov RR, Caron MG, Izquierdo I, Cammarota M, Prado VF, Prado MA (2009a) Reduced expression of the vesicular acetylcholine transporter causes learning deficits in mice. Genes Brain Behav 8:23–35.

de Castro BM, De Jaeger X, Martins-Silva C, Lima RD, Amaral E, Menezes C, Lima P, Neves CM, Pires RG, Gould TW, Welch I, Kushmerick C, Guatimosim C, Izquierdo I, Cammarota M, Rylett RJ, Gomez MV, Caron MG, Oppenheim RW, Prado MA, Prado VF (2009b) The vesicular acetylcholine transporter is required for neuromuscular development and function. Mol Cell Biol 29:5238–5250.

Eiden LE (1998) The cholinergic gene locus. J Neurochem 70:2227–2240.

Erickson JD, Varoqui H, Schäfer MK, Modi W, Diebler MF, Weihe E, Rand J, Eiden LE, Bonner TI, Usdin TB (1994) Functional identification of a vesicular acetylcholine transporter and its expression from a “cholinergic” gene locus. J Biol Chem 269:21929–21932.

Fagergren P, Overstreet DH, Goiny M, Hurd YL(2005) Blunted response to cocaine in the Flinders hypercholinergic animal model of depression. Neuroscience 132:1159–1171.

Fletcher PJ, Soko AD, Higgins GA (2013) Impulsive action in the 5-choice serial reaction time test in 5-HT(2)c receptor null mutant mice. Psychopharmacology 226:561–570.

Gradinaru V, Thompson KR, Zhang F, Mogri M, Kay K, Schneider MB, Deisseroth K (2007) Targeting and readout strategies for fast optical neural control in vitro and in vivo. J Neurosci 27:14231–14238.

Gu Z, Yakel JL (2011) Timing-dependent septal cholinergic induction of dynamic hippocampal synaptic plasticity. Neuron 71:155–165.

Gu Z, Lamb PW, Yakel JL (2012) Cholinergic coordination of presynaptic and postsynaptic activity induces timing-dependent hippocampal synaptic plasticity. J Neurosci 32:12337–12348.

Guzman MS, De Jaeger X, Raulic S, Souza IA, Li AX, Schmid S, Menon RS, Gainetdinov RR, Caron MG, Bartha R, Prado VF, Prado MA(2011) Elimination of the vesicular acetylcholine transporter in the striatum reveals regulation of behaviour by cholinergic-glutamatergic co-transmission. PLoS Biol 9:e1001194.

Guzman MS, De Jaeger X, Drangova M, Prado MA, Gros R, Prado VF(2013) Mice with selective elimination of striatal acetylcholine release are lean, show altered energy homeostasis and changed sleep/wake cycle. J Neurochem 124:658–669.

Harati H, Barbelivien A, Cosquer B, Majchrzak M, Cassel JC(2008) Selective cholinergic lesions in the rat nucleus basalis magnocellularis with limited damage in the

medial septum specifically alter attention performance in the five-choice serial reaction time task. Neuroscience 153:72–83.

Hasselmo ME, Sarter M(2011) Modes and models of forebrain cholinergic neuromodulation of cognition. Neuropsychopharmacology 36:52–73.

Hasselmo ME, Stern CE(2006) Mechanisms underlying working memory for novel information. Trends Cogn Sci 10:487–493.

Holmes A, Wrenn CC, Harris AP, Thayer KE, Crawley JN(2002) Behavioral profiles of inbred strains on novel olfactory, spatial and emotional tests for reference memory in mice. Genes Brain Behav 1:55–69.

Humpston CS, Wood CM, Robinson ES(2013) Investigating the roles of different monoamine transmitters and impulse control using the 5-choice serial reaction time task. J Psychopharmacol 27:213–221.

Janowsky DS, el-Yousef MK, Davis JM, Sekerke HJ(1972) A cholinergic-adrenergic hypothesis of mania and depression. Lancet 2:632–635.

Kalmbach A, Hedrick T, Waters J(2012) Selective optogenetic stimulation of cholinergic axons in neocortex. J Neurophysiol 107:2008–2019.

Lara A, Damasceno DD, Pires R, Gros R, Gomes ER, Gavioli M, Lima RF, Guimarães D, Lima P, Bueno CR Jr., Vasconcelos A, Roman-Campos D, Menezes CA, Sirvente RA, Salemi VM, Mady C, Caron MG, Ferreira AJ, Brum PC, Resende RR, et al. (2010) Dysautonomia due to reduced cholinergic neurotransmission causes cardiac remodeling and heart failure. Mol Cell Biol 30:1746–1756.

Lund D, Ruggiero AM, Ferguson SM, Wright J, English BA, Reisz PA, Whitaker SM, Peltier AC, Blakely RD(2010) Motor neuron-specific overexpression of the presynaptic choline transporter: impact on motor endurance and evoked muscle activity. Neuroscience 171:1041–1053.

Martins-Silva C, De Jaeger X, Guzman MS, Lima RD, Santos MS, Kushmerick C, Gomez MV, Caron MG, Prado MA, Prado VF(2011) Novel strains of mice deficient for

the vesicular acetylcholine transporter: insights on transcriptional regulation and control of locomotor behavior. PloS One 6:e17611.

Martyn AC, De Jaeger X, Magalhães AC, Kesarwani R, Gonçalves DF, Raulic S, Guzman MS, Jackson MF, Izquierdo I, Macdonald JF, Prado MA, Prado VF

(2012) Elimination of the vesicular acetylcholine transporter in the forebrain causes hyperactivity and deficits in spatial memory and long-term potentiation. Proc Natl Acad Sci U S A 109:17651–17656.

McGaughy J, Dalley JW, Morrison CH, Everitt BJ, Robbins TW(2002) Selective behavioral and neurochemical effects of cholinergic lesions produced by intrabasalis infusions of 192 IgG-saporin on attentional performance in a five-choice serial reaction time task. J Neurosci 22:1905–1913.

Naciff JM, Misawa H, Dedman JR(1997) Molecular characterization of the mouse vesicular acetylcholine transporter gene. Neuroreport 8:3467–3473.

Nagode DA, Tang AH, Karson MA, Klugmann M, Alger BE(2011) Optogenetic release of ACh induces rhythmic bursts of perisomatic IPSCs in hippocampus. PloS One 6:e27691.

Nagy PM, Aubert I(2012) Overexpression of the vesicular acetylcholine transporter increased acetylcholine release in the hippocampus. Neuroscience 218:1–11.

Overstreet DH (1993) The Flinders sensitive line rats: a genetic animal model of depression. Neurosci Biobehav Rev 17:51–68.

Overstreet DH, Janowsky DS, Gillin JC, Shiromani PJ, Sutin EL (1986) Stress-induced immobility in rats with cholinergic supersensitivity. Biol Psychiatry 21:657–664.

Parikh V, Kozak R, Martinez V, Sarter M (2007) Prefrontal acetylcholine release controls cue detection on multiple timescales. Neuron 56:141–154.

Parikh V, St Peters M, Blakely RD, Sarter M (2013) The presynaptic choline transporter imposes limits on sustained cortical acetylcholine release and attention. J Neurosci 33:2326–2337.

Patel JC, Rossignol E, Rice ME, Machold RP (2012) Opposing regulation of dopaminergic activity and exploratory motor behavior by forebrain and brainstem cholinergic circuits. Nat Commun 3:1172.

Patil SS, Sunyer B, Höger H, Lubec G (2009) Evaluation of spatial memory of C57BL/6J and CD1 mice in the Barnes maze, the Multiple T-maze and in the Morris water maze. Behav Brain Res 198:58–68.

Picciotto MR, Higley MJ, Mineur YS (2012) Acetylcholine as a neuromodulator: cholinergic signaling shapes nervous system function and behavior. Neuron 76:116–129.

Prado VF, Martins-Silva C, de Castro BM, Lima RF, Barros DM, Amaral E, Ramsey AJ, Sotnikova TD, Ramirez MR, Kim HG, Rossato JI, Koenen J, Quan H, Cota VR, Moraes MF, Gomez MV, Guatimosim C, Wetsel WC, Kushmerick C, Pereira GS, et al. (2006) Mice deficient for the vesicular acetylcholine transporter are myasthenic and have deficits in object and social recognition. Neuron 51:601–612.

Prado VF, Roy A, Kolisnyk B, Gros R, Prado MA (2013) Regulation of cholinergic activity by the vesicular acetylcholine transporter. Biochem J 450:265–274.

Rabenstein RL, Caldarone BJ, Picciotto MR (2006) The nicotinic antagonist mecamylamine has antidepressant-like effects in wild-type but not beta2- or alpha7-nicotinic acetylcholine receptor subunit knockout mice. Psychopharmacology 189:395–401.

Ren J, Qin C, Hu F, Tan J, Qiu L, Zhao S, Feng G, Luo M (2011) Habenula “cholinergic” neurons co-release glutamate and acetylcholine and activate postsynaptic neurons via distinct transmission modes. Neuron 69:445–452.

Robbins TW (2002) The 5-choice serial reaction time task: behavioural pharmacology and functional neurochemistry. Psychopharmacology 163:362–380.

Rocha-Resende C, Roy A, Resende R, Ladeira MS, Lara A, de Morais Gomes ER, Prado VF, Gros R, Guatimosim C, Prado MA, Guatimosim S (2012) Non-neuronal

cholinergic machinery present in cardiomyocytes offsets hypertrophic signals. J Mol Cell Cardiol 53:206–216.

Roghani A, Feldman J, Kohan SA, Shirzadi A, Gundersen CB, Brecha N, Edwards RH (1994) Molecular cloning of a putative vesicular transporter for acetylcholine. Proc Natl Acad Sci U S A 91:10620–10624.

Romberg C, Mattson MP, Mughal MR, Bussey TJ, Saksida LM (2011) Impaired attention in the 3xTgAD mouse model of Alzheimer's disease: rescue by donepezil (Aricept) J Neurosci 31:3500–3507.

Roy A, Lara A, Guimarães D, Pires R, Gomes ER, Carter DE, Gomez MV, Guatimosim S, Prado VF, Prado MA, Gros R (2012) An analysis of the myocardial transcriptome in a mouse model of cardiac dysfunction with decreased cholinergic neurotransmission. PLoS One 7:e39997.

Sarter M, Hasselmo ME, Bruno JP, Givens B (2005) Unraveling the attentional functions of cortical cholinergic inputs: interactions between signal-driven and cognitive modulation of signal detection. Brain Res Brain Res Rev 48:98–111.

Sarter M, Gehring WJ, Kozak R (2006) More attention must be paid: the neurobiology of attentional effort. Brain Res Rev 51:145–160.

Song H, Ming G, Fon E, Bellocchio E, Edwards RH, Poo M (1997) Expression of a putative vesicular acetylcholine transporter facilitates quantal transmitter packaging. Neuron 18:815–826.

Varvel SA, Lichtman AH (2002) Evaluation of CB1 receptor knockout mice in the Morris water maze. J Pharmacol Exp Ther 301:915–924.

Vorhees CV, Williams MT (2006) Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nat Protoc 1:848–858.

Wess J, Eglen RM, Gautam D (2007) Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development. Nat Rev Drug Discov 6:721–733.

Witten IB, Lin SC, Brodsky M, Prakash R, Diester I, Anikeeva P, Gradinaru V, Ramakrishnan C, Deisseroth K (2010) Cholinergic interneurons control local circuit activity and cocaine conditioning. Science 330:1677–1681.

Zhao S, Ting JT, Atallah HE, Qiu L, Tan J, Gloss B, Augustine GJ, Deisseroth K, Luo M, Graybiel AM, Feng G (2011) Cell type-specific channelrhodopsin-2 transgenic mice for optogenetic dissection of neural circuitry function. Nat Methods 8:745–752

Chapter 3

Forebrain deletion of the vesicular acetylcholine transporter results in deficits in executive function, metabolic, and RNA splicing abnormalities in the prefrontal cortex

3.1 Chapter Summary

One of the key brain regions in cognitive processing and executive function is the prefrontal cortex (PFC), which receives cholinergic input from basal forebrain cholinergic neurons. We evaluated the contribution of synaptically released acetylcholine (ACh) to executive function by genetically targeting the vesicular acetylcholine transporter (VAChT) in the mouse forebrain. Executive function was assessed using a pairwise visual discrimination paradigm and the 5-choice serial reaction time task (5-CSRT). In the pairwise test, VAChT-deficient mice were able to learn, but were impaired in reversal learning, suggesting that these mice present cognitive inflexibility. Interestingly, VAChT-targeted mice took longer to reach criteria in the 5-CSRT. Although their performance was indistinguishable from that of control mice during low attentional demand, increased attentional demand revealed striking deficits in VAChT-deleted mice. Galantamine, a cholinesterase inhibitor used in Alzheimer's disease, significantly improved the performance of control mice, but not of VAChT-deficient mice on the 5-CSRT. *In vivo* magnetic resonance spectroscopy showed altered levels of two neurochemical markers of neuronal function, taurine and lactate, suggesting altered PFC metabolism in VAChT-deficient mice. The PFC of these mice displayed a drastic reduction in the splicing factor heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNPA2/B1), whose cholinergic-mediated reduction was previously demonstrated in Alzheimer's disease. Consequently, several key hnRNPA2/B1 target transcripts involved in neuronal function present changes in alternative splicing in VAChT-deficient mice, including pyruvate kinase M, a key enzyme involved in lactate metabolism. We propose that VAChT-targeted mice can be used to model and to dissect the neurochemical basis of executive abnormalities.

3.2 Introduction

The prefrontal cortex (PFC) is essential for the modulation of executive function, which is loosely defined as a set of cognitive tools that allows hierarchical and timely control of actions leading to specific behaviors (Alvarez and Emory, 2006; Robbins and Roberts, 2007; Chudasama, 2011). Disruption in executive function is a key symptom in neurological and neuropsychiatric disorders, including Alzheimer's disease (AD; Perry and Hodges, 1999; Traykov et al., 2007; McGuinness et al., 2010), schizophrenia (Morice, 1990), autism (Hill, 2004; Sala et al., 2011), and drug addiction (Stalnaker et al., 2009). The basic neurochemical underpinnings of executive function are, however, still poorly understood.

Cholinergic deficits are a hallmark of AD (Perry et al., 1977; Whitehouse et al., 1981, 1982). Moreover, amyloid β (Aβ) oligomers, potential toxins in AD, disrupt cholinergic synaptic transmission in the PFC (Chen et al., 2013). Cholinergic deficiency in AD can have widespread effects, including global changes in alternative splicing of genes involved in synaptic plasticity (Berson et al., 2012).

Acetylcholine (ACh) in the PFC has been implicated in controlling attention (Elliott, 2003; Jurado and Rosselli, 2007), one of the components of executive function. Cue detection and top-down modulation of attentive behavior have both been shown to activate PFC cholinergic activity and to be modulated by cholinergic signaling (Sarter et al., 2001; Parikh et al., 2007). Cholinergic transients in the PFC have been linked to cue detection, and both tonic and phasic PFC ACh release seem to regulate attentional demand (Parikh et al., 2007; for review, see Hasselmo and Sarter, 2011), which may depend mainly on nicotinic receptor signaling (McGaughy et al., 1999; Grottick and Higgins, 2000; Parikh et al., 2010; Guillem et al., 2011).

Cognitive flexibility, the ability to alter strategy according to changing environmental cues, is another key component of executive function (Elliott, 2003; Jurado and Rosselli, 2007). The neurochemical basis of cognitive flexibility is not fully understood, but serotonin is thought to play critical roles (Schmitt et al., 2006; Evers et al., 2007; Brigman et al., 2010).

Release of ACh is a tightly regulated process, with the vesicular acetylcholine transporter (VAChT) controlling a limiting key step (de Castro et al., 2009a; Kolisnyk et al., 2013; for review, see Prado et al., 2013). Genetic elimination of VAChT from the forebrain causes deficits in reversal learning assessed using the Morris Water Maze (MWM) (Martyn et al., 2012). Deficits in reversal learning may be related to hippocampal dysfunction, but could also reflect alterations in behavioral flexibility. To examine if decreased levels of VAChT, a change observed in AD (Efange et al., 1997; Chen et al., 2011), affects executive function we have used touchscreen tasks. We report that elimination of forebrain VAChT caused severe deficits in cognitive flexibility and in sustained attention. In addition, we found that these mutant mice have profound changes in RNA processing in the PFC, which correlate with behavioral and metabolic deficits. Our results suggest that elimination of forebrain cholinergic activity in mice provides a model for understanding the neurochemical basis of executive function.

3.3 Material and Methods

3.3.1Animals

Generation of VAChTSix3-Cre-flox/flox mice was previously described (Martyn et al., 2012). In short, VAChTSix3-Cre-flox/flox mice were generated by crossing VAChTflox/flox (mixed C57BL/6J × 129/SvEv background, backcrossed to C57BL/6J for five generations) with the Six3-Cre mouse line (NMRI background, backcrossed to C57BL/6J for five generations). We then intercrossed VAChTSix3-Cre-flox/wt mice to obtain VAChTSix3-Cre-flox/flox. For the galantamine experiments, the mice used were wild-type C57BL/6J. Mice were housed in groups of three or four per cage without environmental enrichment in a temperature-controlled room with 14/10 h light/dark cycle, and water was provided ad libitum. Only male mice were used in these studies. Mice were restricted to 85% of their free-feed weight and maintained on 85% of their weight for the duration of the study. All procedures were conducted in accordance with guidelines from the Canadian Council of Animal Care at the University of Western Ontario with an approved institutional animal protocol (2008–127).

3.3.2 Western Blotting

Mouse PFC was collected, protein was isolated, and immunoblotting was performed as previously described (Martins-Silva et al., 2011). The antibodies used were anti-VAChT (catalog #139103; Synaptic Systems) at a 1:3000 dilution, anti-Synaptophysin (catalog #S5768; Sigma-Aldrich) at a 1:500 dilution, anti-hnRNP A2/B1 (catalog #sc-10035; Santa Cruz Biotechnology) at a 1:500 dilution, and anti-β-Actin (catalog #ab49900; Abcam), at a 1:15000 dilution. Band intensity was quantified using FluoroChemQ software (Thermo Fisher Scientific).

3.3.3 Acetylcholine Release

ACh release from prefrontal cortical brain slices was quantified by labeling slices with [3H] methyl-choline before using KCl to stimulate release of labeled ACh as previously described (Guzman et al., 2011).

3.3.4 qPCR

To measure mRNA expression, total RNA was extracted from freshly dissected PFC tissue, using the Aurum Total RNA for fatty and fibrous tissue kit (Bio-Rad) according to the manufacturer's instructions. cDNA synthesis and qPCR analysis were performed as previously described (Guzman et al., 2011). Primer sequences used to determine alternative spliced transcripts are found in Table 3.1. β-Actin was used as a reference transcript for all reactions. For alternative splicing experiments, the alternative exon levels were normalized to a constitutively expressed exon from the same gene.

Table 3.1. Primers for alternative splicing assay

Target	Forward primer(5′ → 3′)	Reverse primer (5′ → 3′)
CD55 exon 8	CCCAGCATGTACCTGTTACC	TCACATGCAAAACTGTCAAGG
CD55 exon 1	TGTCTCTGTTGCTGCTGTCC	TGCTCAGCAAACTTGGAGTG
DRAM2 exon 2	TGATTCAAGGTTCACACTCACA	AAAACTGAGGCCTTGCTGAA
DRAM2 exon 4	TTCAGCAAGGCCTCAGTTTT	TCAGGAGGTATTGTCCCTGTG
SIPA1L1 intron 5	TCAGGCATGCAGTTCTTTTG	GAAAGCAGGCAGTACCTTCG
SIPA1L1 exon 4	TAGTGTGGACGCTGCTGTCT	GGCTCTGTGGTCACCAGAAT
DYSTONIN exon 41	ATGGCATTTCCCCCATTAG	GGAGGTTGGTTTTGCTTCAA
DYSTONIN exon 7	GAGCGGGACAAAGTTCAAAA	CCCGTCCCTCAGATCCTC
REELIN exon 3	ATCATGTCCGACCACCAGTT	ATCATGTCCGACCACCAGTT
REELIN exon 18	GCAGTGCCAGACTTTCCTCT	GCCTCCCATCTTTGTTGAAA
REELIN exon 1	GGCAACCCCACCTACTACG	GACTGGATGCTTGTCGAGGT
mENAH	CGGCAGTAAGTCACCTGTCA	C TTCAGCTTTGCCAGCTCTT
mENAH invasive	GATTCAAGACCATCAGGTTGTG	CAATGTTGGCCCTAAATAGAA
PKM1	CATGCAGCACCTGATAGCTC	TTATAAGAGGCCTCCACGCTG
PKM2	GCAGTGGGGCCATTATCGT	GGGATTTCGAGTCACGGCAA

3.3.5 Magnetic resonance imaging

Magnetic resonance spectroscopy of the prefrontal region was performed in four VAChTSix3-Cre-flox/flox mice and four littermates (VAChTflox/flox). Spectroscopic localization of a 24 µl voxel was achieved by adiabatic selective refocusing (DelaBarre and Garwood, 1998) on a 9.4 tesla horizontal bore small animal Agilent magnetic resonance imaging (MRI) scanner. Water-suppressed full spectra (TR/TE = 3250/20 ms, 128 acquisitions), water-suppressed macromolecule spectra (TR/TE = 5000/20 ms, inversion time TI = 873 ms, 128 acquisitions), and water-unsuppressed spectra (TR/TE = 3250/20 ms, 8 acquisitions) were acquired. All animals were anesthetized with 2% isoflurane during the procedure and were maintained at 37° by blowing warm air into the bore of the magnet using a Model 1025 Small Animal Monitoring and Gating System (SA Instruments).

3.3.6 Metabolite analysis

Magnetic resonance spectra were analyzed using purpose-built software (fitMAN; Bartha et al., 1999) to determine the contribution of each metabolite relative to total creatine as previously described (Bartha, 2007, 2008). Briefly, spectra were lineshape corrected (Bartha et al., 2000b) and the macromolecule and residual water contribution was removed (Kassem and Bartha, 2003). Then, the spectrum was fitted in the time domain to a basis set of 19 metabolite lineshapes (Pfeuffer et al., 1999; Bartha et al., 2000a). Five metabolites (measured relative to creatine) were reliably measured and included in group comparisons: N-acetylaspartate (NAA), myo-inositol (Myo), choline (Cho), taurine (Tau), and lactate (Lac).

3.3.7 Touchscreen behavioral assessment

3.3.7.1 Apparatus and task

Mice were trained in the 5-choice serial reaction time task (5-CSRT) and in the pairwise visual discrimination in automated Bussey–Saksida Mouse Touchscreen Systems

model 81426 (Campden Instruments). Schedules were designed and data were collected using the ABET II Touch software v.2.15 (Lafayette Instruments).

3.3.7.2 Pretraining

Before being trained on the pairwise visual discrimination or the 5-CSRT task, mice were first put through a pretraining program. This consisted of first habituating the mice to the testing chamber with the lights off for 15 min. The next day, mice were left in the chamber with the lights off for 20 min, this time with the reward tray primed with a 150 µl reward (strawberry milkshake; Saputo Dairy Products), and a tone was played whenever the mouse entered the reward tray. Whenever the mouse returned to the reward tray, it received a reward (7 µl) paired with the tone. This training was repeated the next 2 d for 40 min sessions (phase 1).

The next training phase (phase 2) involved pairing the reward with the presentation of the stimulus on the touchscreen. The stimulus appeared randomly in one of the windows and after 30 s, it was removed and a reward (7 µl) was given paired with a tone. If the mouse touched the screen while the image was displayed, it immediately received a reward (7 µl). Once the mouse collected the reward a new trial was initiated. This phase was repeated until the mouse completed 30 trials within 60 min (phase 2).

Phase 3 was used to further shape behavior. It involved displaying the stimulus randomly in one of the windows. The mouse was required to touch the stimulus on the screen to receive a reward (7 µl) paired with a tone. There was no response to the mouse touching anything but the stimulus. Once again, this was repeated until the mouse completed 30 trials within 60 min. Phase 4 was identical to phase 3 except that the mouse was required to initiate each trial by nose poking the reward tray. Criterion was 30 correct trials within 60 min.

Finally, in the last pretraining phase (phase 5), the previous procedure was repeated but if the mouse touched an incorrect screen, it received a 5 s time-out, during which the light was turned on. The final phase had a stricter criterion, requiring the mouse to perform 30 trials in 60 min with at least 23 correct responses in 2 consecutive days.

3.3.7.3 Pairwise visual discrimination and reversal

Pairwise visual discrimination and reversal task were performed as previously described (Romberg et al., 2013). Mice used in this experiment were 6–8 months old. At the beginning of each session, the reward tray was primed with 7 µl of milkshake. Briefly, the mice were first trained to discriminate between two visual stimuli, which were presented simultaneously, with their spatial location randomized over a 30 trial session. If mouse nose poked the correct stimulus (S+), a tone was played and mouse received a reward (7 µl), whereas if the incorrect stimulus (S−) was nose poked, light in the chamber was turned on for 5 s (time-out) followed by a correction trial. During the correction trial, the trial was repeated until the mouse poked the correct stimulus. Criterion was reached when the mouse selected the correct image (S+) on 80% of the trials, excluding correction trials, for 2 consecutive days. Once mice reached criteria, they were given two sessions to assess baseline performance on the task. For reversal learning the rule associated to each stimulus was switched, that is, the rewarded image (S+) during acquisition became the (S−) image in reversal and was punished, while the (S−) image from acquisition became the correct stimulus and was rewarded. Reversal learning was assessed over the course of 10 sessions.

3.3.7.4 Training in 5-CSRT

The 5-CSRT task was performed as previously described (Romberg et al., 2011). A distinct cohort of mice (8–10 months old) was trained in the 5-CSRT task. At the beginning of each session, the reward tray was primed with 7 µl of milkshake. Each trial was initiated after the mouse nose poked the magazine. In this phase, the stimulus was delivered after a variable 5–10 s delay (delay period), during which the animal was required to attend to the screen. In case the mouse prematurely touched the screen during this delay, the response was recorded as premature and the mouse was punished with a 10 s time-out. The stimulus duration was initially set to 4 s, followed by a limited holding period of 5 s, during which the stimulus was absent, but the mouse could still respond to the location (holding period). Responses to the stimulus window during stimulus presence or the holding period were recorded as correct and a 7 µl

reward was administered, while responses to any other window were recorded as incorrect. A correct choice was rewarded with a tone and food delivery. An incorrect response was punished with a 10 s time-out. A failure to respond to any window both during stimulus display, or during the holding period, was recorded as an omission and punished with a 10 s time-out. Perseverative responses to the screen after premature, correct, and incorrect choices were also recorded. Once the performance of a mouse reached criterion at 4 s stimulus duration (80% accuracy, 20% omissions for 3 consecutive days), the stimulus duration was reduced to 2 s. After reaching criterion with the 2 s stimulus, mice were tested for two more days. The mean measurement of those 2 d was used to assess baseline performance. If the mouse completed <30 trials, it was considered not to have reached criteria, even if it met accuracy and omissions thresholds.

3.3.7.5 Probe trial

To probe attention in mice we used a previously described probe trial schedule with reduced stimulus durations (Romberg et al., 2011). Briefly, each mouse was tested for 2 consecutive days at a given stimulus duration (1.5, 1, 0.8, and 0.6 s). After each test, the animal was retested onto the 2 s stimulus duration for 2 d or until criteria were reached (80% accuracy, 20% omission), to assess baseline performance. The order of the probe trial sessions were semi-randomized using a Latin square method.

3.3.7.6 Distraction task

To further test attentional demand, and assess susceptibility to distraction, we developed a distraction version of the 5-CSRT task. During this task, a 1000 ms distractor tone, different from the reward tone, was played semi-randomly at five different time points during the delay period: 0 (corresponding to when the mouse initiates the task), 0.5, 2.5, 4.5, and 5 s (corresponding to when the stimulus is displayed on the touchscreens). Stimulus duration was set to 2 s. For each distractor tone time point mice were tested for five sessions. At the end of the five sessions one baseline task (50 trial, 2 s stimulus duration, no distractor sounds) was performed before moving to the next distractor time point.

3.3.7.7 5-CSRT task measures

On all 5-CSRT task sessions, accuracy was defined as the total number of correct responses, divided by the number of correct and incorrect (touches to a wrong window while the correct stimulus was still displayed) responses. The rate of omissions was the proportion of omitted responses to total trials. Response latency was the time for the mouse to touch the correct stimulus after its onset. Reward collection latency was the time for the mouse to return to the reward tray once it had touched the correct stimulus. A premature response was counted when the mouse touched one of the windows before stimulus onset. Finally, a perseverative response was any identical response that occurred following a correct, incorrect, or premature response.

3.3.7.8 Drug treatments

Galantamine hydrobromide, a cholinesterase inhibitor, (Sigma-Aldrich) was dissolved in physiological saline (0.9% NaCl) before administration. Sixty minutes before being tested on the 5-CSRT mice received an intraperitoneal injection of drug (100 µl, 1 mg/kg) or saline. The dose for galantamine was selected based on previous studies (Prado et al., 2006; de Castro et al., 2009b; De Jaeger et al., 2013). Previously, we have tested 3 mg/kg galantamine in other tasks, but this dose was no more effective than 1 mg/kg and it produced hypersalivation in mice (de Castro et al., 2009b). Moreover, at 1 mg/kg wild-type mice were able to increase their performance in the 5-CSRT (see below). Mice were tested at the 0.6 s stimulus duration, which represents a high attention demanding task (Romberg et al., 2011). The order of the injections was counterbalanced. Between injections mice had three washout days wherein their performance on the 5CSRT was re-baselined at the 2 s stimulus duration.

3.3.8 Statistical analysis

For the pairwise visual discrimination task response accuracy was calculated as the number of correct trials divided by the total number of trials, excluding correction trials. Data are expressed as mean ± SEM. SigmaStat 3.5 software was used for statistical analysis. Comparison between two experimental groups was made by Student's t test or

Mann–Whitney rank sum test, when the data did not follow a normal distribution. When several experimental groups or treatments were analyzed, two-way ANOVA or two-way ANOVA with repeated measures was used as required. When appropriate, a Tukey's HSD post hoc comparison test was used. In all comparisons, $p < 0.05$ was considered statistically significant.

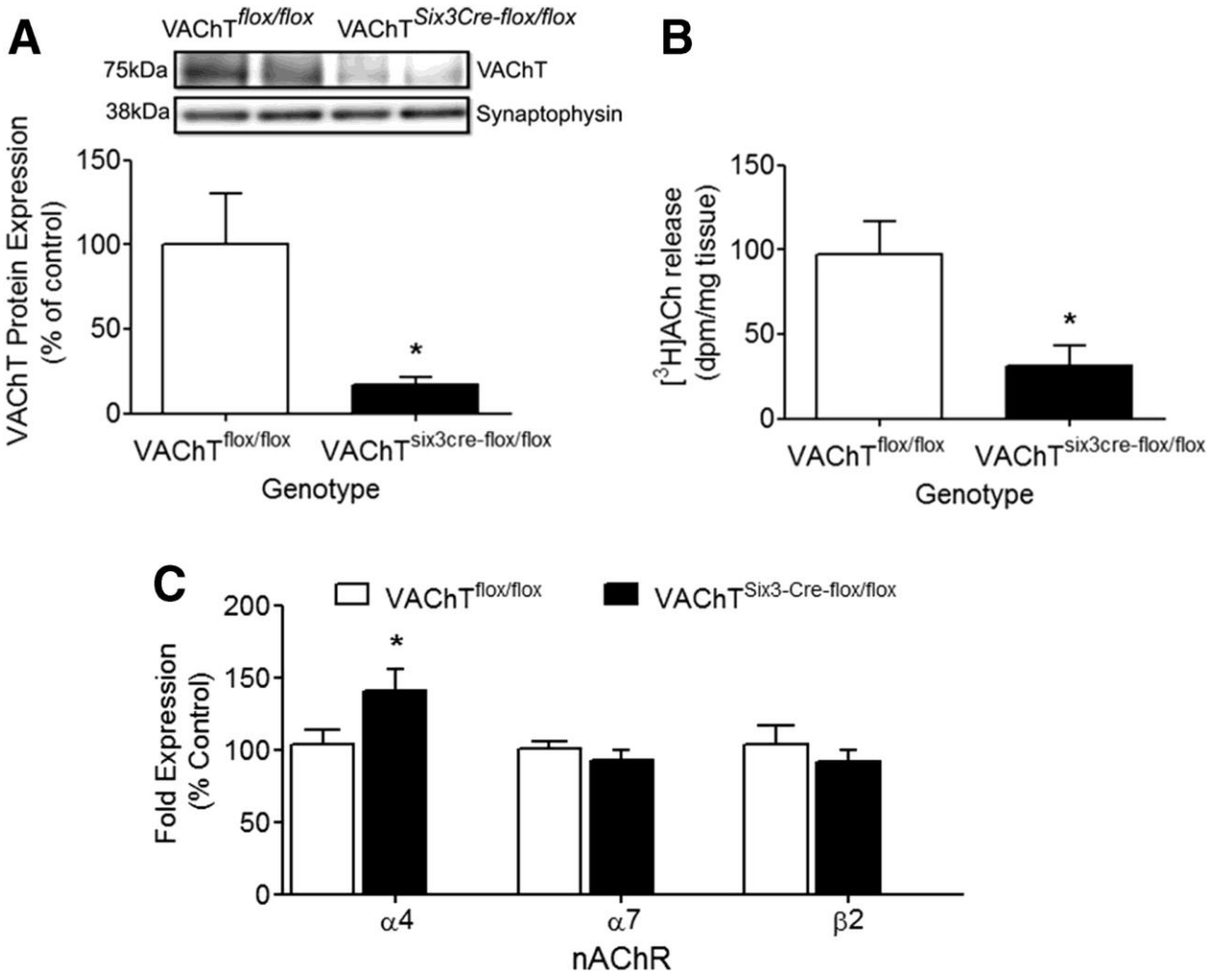
3.4 Results

3.4.1VAChTSix3-Cre-flox/flox mice have reduced VAChT and ACh release in the PFC

We have previously reported that VAChTSix3-Cre-flox/flox mice have the VAChT gene deleted from >90% of their basal forebrain cholinergic neurons (Martyn et al., 2012). To confirm that this deletion affected prefrontal cortical cholinergic signaling, we performed Western blot analysis to assess VAChT expression. VAChTSix3-Cre-flox/flox mice have a significant reduction in PFC VAChT protein expression ($t(6) = 2.706$, $p = 0.0353$; Fig. 5.1A). Moreover, this reduction in VAChT protein levels results in a significant decrease in newly synthesized [3H] ACh release in slices of PFC from VAChTSix3-Cre-flox/flox mice when compared with control mice ($t(4) = 2.899$, $p = 0.0442$; Fig. 5.1B). We used qPCR to assess the expression of nicotinic receptors (nAChRs), which have been shown previously to be critical for attentional performance. The PFC of VAChTSix3-Cre-flox/flox mice shows no significant change in the expression of α7nAChR mRNA ($t(10) = 0.9300$, $p = 0.3743$) nor β2nAChR mRNA ($t(10) = 0.8359$, $p = 0.4227$). Interestingly, α4nAChR mRNA is slightly upregulated ($t(10) = 2.550$, $p = 0.0289$; Fig. 5.1 C).

Figure 3.1 Expression of VAChT in the PFC of VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice. ***A***, VAChT protein expression in the PFC with representative immunoblots inset (*n* = 4). ***B***, Release of newly synthesized [^{3}H]ACh in PFC slices (*n* = 3). ***C***, qPCR analysis of nAChR expression (*n* = 6, data are mean ± SEM, **p* < 0.05).

Figure 3.1 Expression of VAChT in the PFC of VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice.



3.4.2 Decreased forebrain cholinergic tone specifically disturbs reversal learning in the pairwise visual discrimination task

We have previously demonstrated that VAChTSix3-Cre-flox/flox mice have impairments in reversal learning in the MWM (Martyn et al., 2012), suggesting the possibility that these mice have behavior flexibility deficits. To determine whether VAChTSix3-Cre-flox/flox mice present alterations in cognitive flexibility we used a “nonhippocampal” pairwise visual discrimination task (Romberg et al., 2013). This task has been previously shown to depend on the PFC and also on striatal-cortical loops (Graybeal et al., 2011). The performance of VAChTSix3-Cre-flox/flox mice did not differ from that of controls (VAChTflox/flox) when they were trained to operate the touchscreen (pretraining phase). The number of sessions the mice took to acquire each training phase did not differ from control (RM-ANOVA, no effect of genotype $F(1,54) = 0.3950$, $p = 0.5398$; main effect of training phase $F(4,54) = 5.227$, $p = 0.0012$; and no interaction effect $F(4,54) = 1.389$, $p = 0.2495$; Figure 3.2A). Acquisition of the pairwise visual discrimination task (Fig. 5.2B; for stimuli used) did not differ between genotypes, in terms of sessions to criteria (Fig. 5.2C; $t(14) = 0.2446$, $p = 0.8117$), correction errors made (Fig. 5.2D; $t(14) = 0.2942$, $p = 0.7746$), response latency (Fig. 5.2E; $t(14) = 1.019$, $p = 0.3256$), or reward collection latency (Fig. 5.2F; $t(14) = 0.2606$, $p = 0.7988$). We have previously reported that these mice are hyperactive in novel environments, but they are able to habituate to the environment (Martyn et al., 2012). Hence, due to the extensive training for the performance in the touchscreen tasks the lack of differences in response and reward collection latencies is not surprising. However, VAChTSix3-Cre-flox/flox mice showed severe reversal learning impairment (Fig. 5.3A; for the stimuli used), measured by the percentage of correct responses (Fig. 5.3B; RM-ANOVA, main effect of genotype $F(1,132) = 19.78$, $p = 0.0008$; main effect of session $F(11,132) = 23.28$, $p = 0.0001$; and significant interaction effect $F(1,12) = 5.035$, $p = 0.0001$) and by its increased correction errors (Fig. 5.3C; RM-ANOVA, main effect of genotype $F(1,132) = 14.72$, $p = 0.0024$; main effect of session $F(11,132) = 14.37$, $p = 0.0001$; and significant interaction effect $F(1,12) = 2.817$, $p = 0.0025$). Post hoc analysis showed that VAChTSix3-Cre-flox/flox mice never improved significantly from the first reversal

session and in 10 sessions they performed only at chance level. Importantly, during the reversal trials, the VAChTSix3-Cre-flox/flox mice did not differ from controls in terms of response latency (Fig. 5.3D; RM-ANOVA, no effect of genotype $F(1,154) = 0.4233$, $p = 0.5258$; main effect of session $F(11,154) = 4.705$, $p = 0.0001$; and no interaction effect $F(11,154) = 0.9997$, $p = 0.4493$) or reward collection latency (Fig. 5.3E; RM-ANOVA, no effect of genotype $F(1,154) = 1.107$, $p = 0.3105$; main effect of session $F(11,132) = 3.965$, $p = 0.0001$; and no interaction effect $F(11,154) = 1.141$, $p = 0.3333$). These results suggest that forebrain cholinergic tone is important for cognitive flexibility.

Figure 3.2. VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice show normal visual discrimination learning. ***A***, Mean number of trials required to reach criteria during the operant conditioning, pretraining phases. ***B***, Image of a mouse performing the task, with the fan shown as the S+ and the marbles as the S−. ***C***, Number of sessions to criteria in the pairwise visual discrimination learning task. ***D***, Correction errors made to achieve discrimination criteria. ***E***, Mean response latency. ***F***, Mean reward collection latency. *n* = 8, data are mean ± SEM.

Figure 3.2. VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice show normal visual discrimination learning.

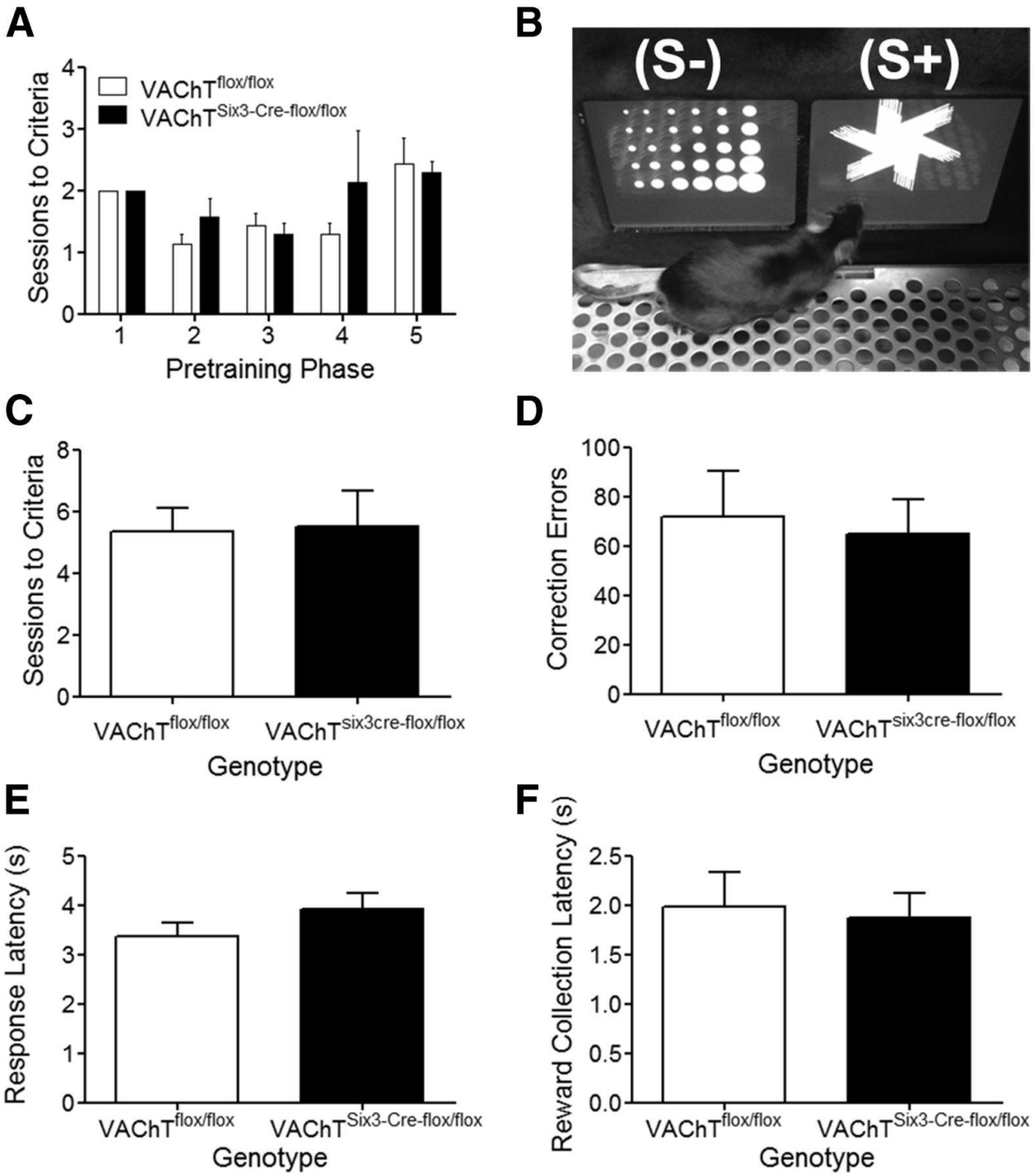
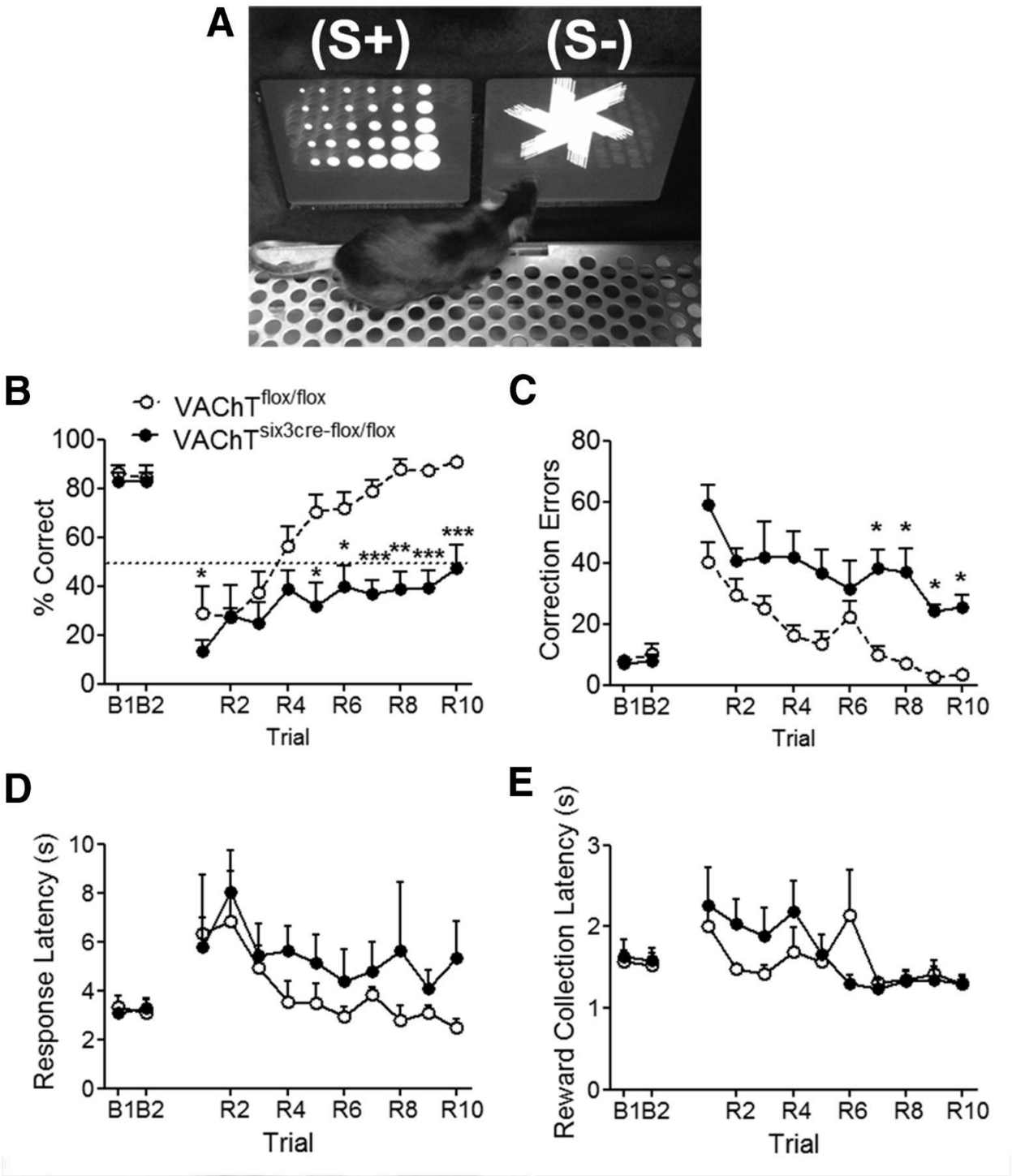


Figure 3.3. VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice have impaired reversal learning. ***A***, Image of a mouse performing the task, this time with the fan shown as the S− and the marbles as the S+. ***B***, Choice accuracy before (B1 and B2) and during reversal trials (R2–R10), dashed line denotes chance (50%). ***C***, Number of correction errors made across reversal trials. ***D***, Response latency across reversal trials. ***E***, Reward collection latency across reversal trials (n = 8, data are mean ± SEM, *p < 0.05, **p < 0.01, and ***p < 0.001).

Figure 3.3. VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice have impaired reversal learning.

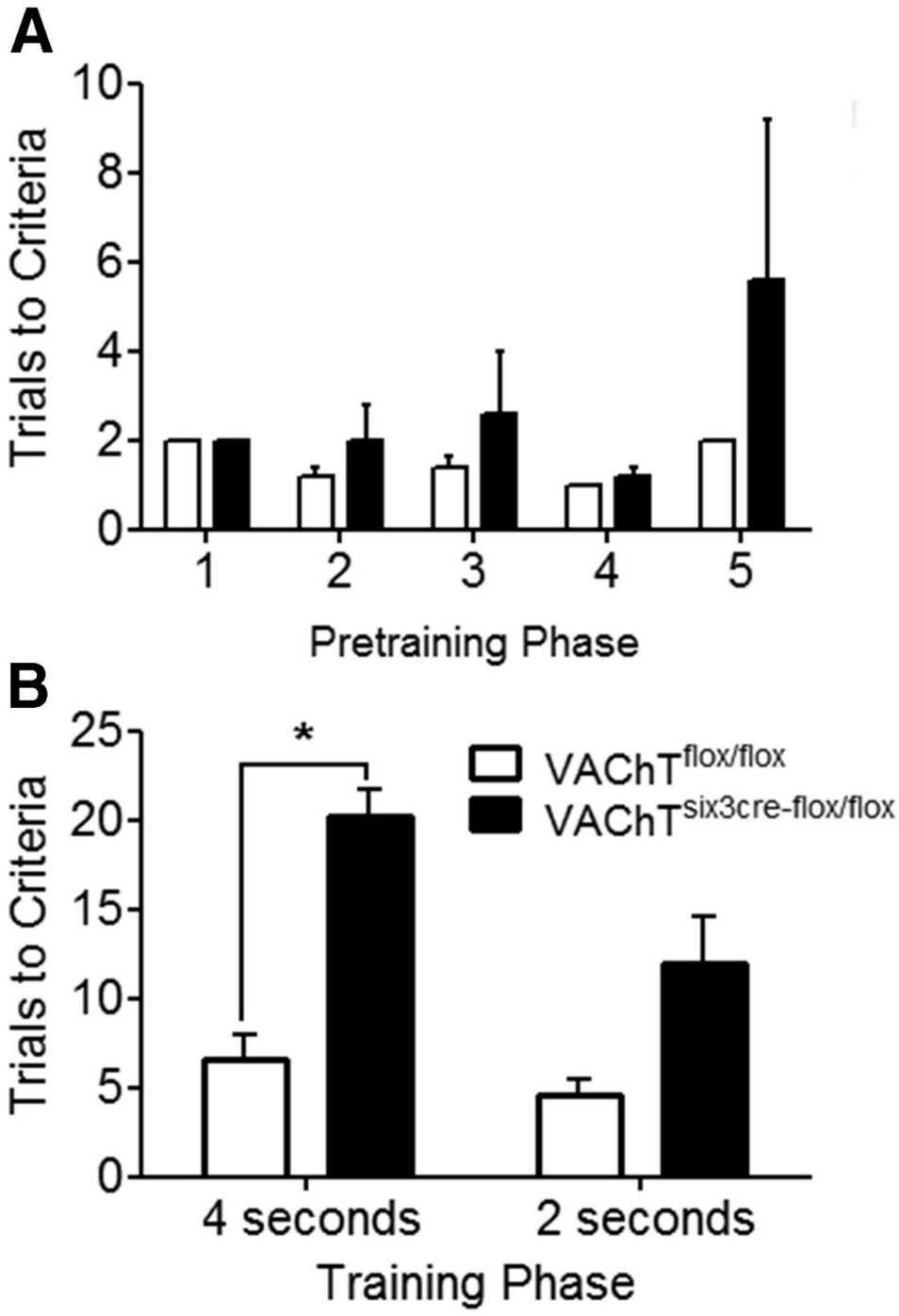


3.4.3 VAChTSix3-Cre-flox/flox mice have impaired acquisition of the 5-CSRT task

Similar to the results for visual discrimination experiments, the performance of the second cohort of VAChTSix3-Cre-flox/flox mice did not differ from that of control mice for touchscreen operation training (pretraining phase). The number of sessions necessary to reach criteria across all pretraining phases did not differ between genotypes (RM-ANOVA, no effect of genotype $F(1,32) = 1.528$, $p = 0.2515$; no effect of training phase $F(4,32) = 1.492$, $p = 0.2280$; Figure 3.4A). However, when mice were trained to respond to flashes of light displayed in one of the five spatial locations on the touchscreen (training phase), VAChTSix3-Cre-flox/flox showed a significantly worse performance, needing more sessions to reach criteria (RM-ANOVA, main effect of genotype, $F(1,8) = 10.06$, $p = 0.0132$; main effect of stimulus duration, $F(1,8) = 5.731$, $p = 0.0436$, no interaction effect, $F(1,8) = 4.252$, $p = 0.0731$; Fig. 5.4B).

Figure 3.4. Training in the 5-CSRT task. ***A***, Mean number of trials required to reach criteria during the operant conditioning, pretraining phases.***B***, Mean number of trials required to reach criteria at 4 and 2 s stimulus duration (n = 6, data are mean ± SEM, *p < 0.05).

Figure 3.4. Training in the 5-CSRT task

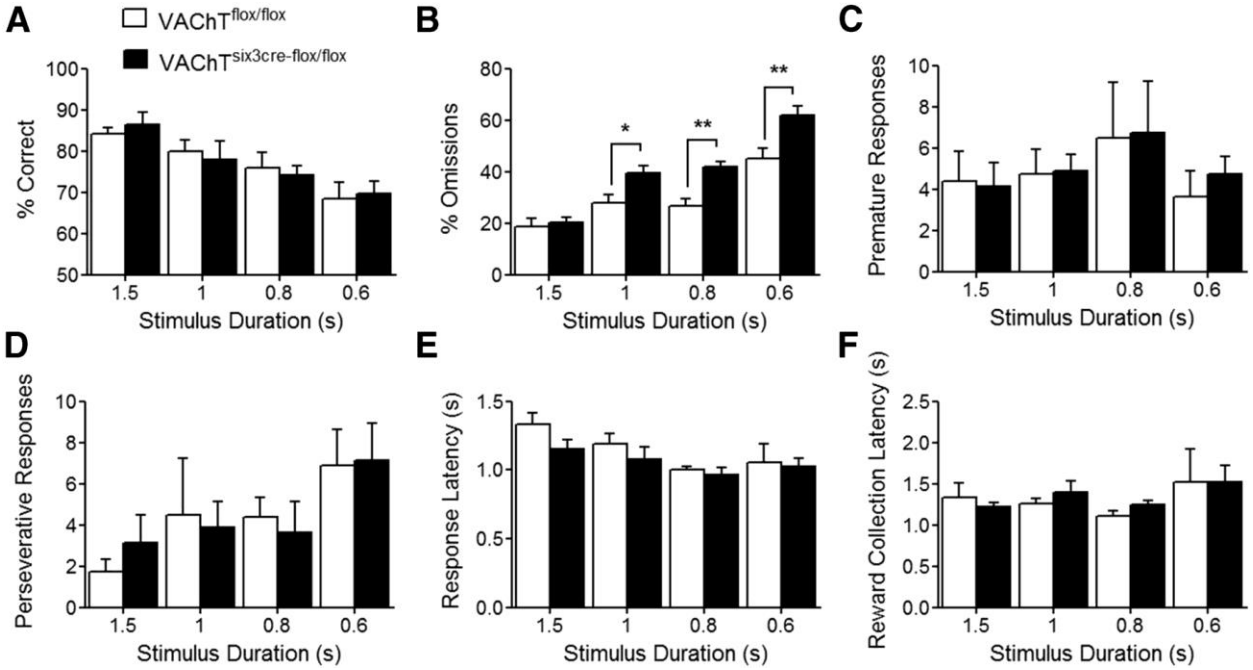


3.4.4 Deletion of forebrain VAChT results in inattentive but not impulsive or compulsive behavior

Once mice reached criteria they were tested for attentional performance by decreasing stimulus duration to 1.5, 1, 0.8, and 0.6 s as previously described (Romberg et al., 2011). At 1.5 s, VAChTSix3-Cre-flox/flox mice were able to perform identically to control mice in both accuracy and omissions (Fig. 5.5A,B). In contrast, increasing attentional demand by diminishing the stimuli period revealed an attentional deficit in VAChTSix3-Cre-flox/flox mice. Response accuracy did not differ between genotypes (RM-ANOVA, no effect of genotype $F(1,24) = 0.0007548$, $p = 0.9788$; main effect of stimulus duration $F(3,24) = 10.28$, $p < 0.001$; Figure 3.5A). However, VAChTSix3-Cre-flox/flox mice presented an increased rate of omission (RM-ANOVA, main effect of genotype $F(1,24) = 57.99$, $p < 0.0001$; main effect of stimulus duration $F(3,24) = 36.44$, $p < 0.0001$; no interaction effect $F(3,24) = 2.176$, $p = 0.1171$; Figure 3.5B). Post hoc analysis revealed that VAChTSix3-Cre-flox/flox mice omitted more at 0.6–1 s stimulus durations. Interestingly, VAChT-deficient mice had no alterations in premature responses (RM-ANOVA, no effect of genotype $F(1,24) = 0.07440$, $p = 0.7874$; no effect of stimulus duration $F(3,24) = 0.9891$, $p = 0.4146$; Figure 3.5C) or perseverative responses (RM-ANOVA, no effect of genotype $F(1,24) = 0.04610$, $p = 0.9695$; no effect of stimulus duration $F(3,24) = 1.244$, $p = 0.3232$; Figure 3.5D). Additionally, mutant mice did not differ from littermate controls in response latency (RM-ANOVA, no effect of genotype $F(1,24) = 2.279$, $p = 0.1818$; no effect of stimulus duration $F(3,24) = 0.09686$, $p = 0.7662$; Figure 3.5E) and reward collection latency (RM-ANOVA, no effect of genotype $F(1,24) = 2.279$, $p = 0.1818$; no effect of stimulus duration $F(3,24) = 1.189$, $p = 0.3420$; Figure 3.5F).

Figure 3.5. VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice have attentional deficits. Performance and response measures during 5-CSRT task probe trials. Mice were subjected to a series of probe trials and the average values of 50 trial sessions are plotted. ***A***, Mean accuracy. ***B***, Mean rate of omissions. ***C***, Mean premature responses. ***D***, Mean perseverative responses. ***E***, Mean response latency. ***F***, Mean reward collection latency (n = 6, data are mean ± SEM, *p < 0.05 and **p < 0.01).

Figure 3.5. VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice have attentional deficits

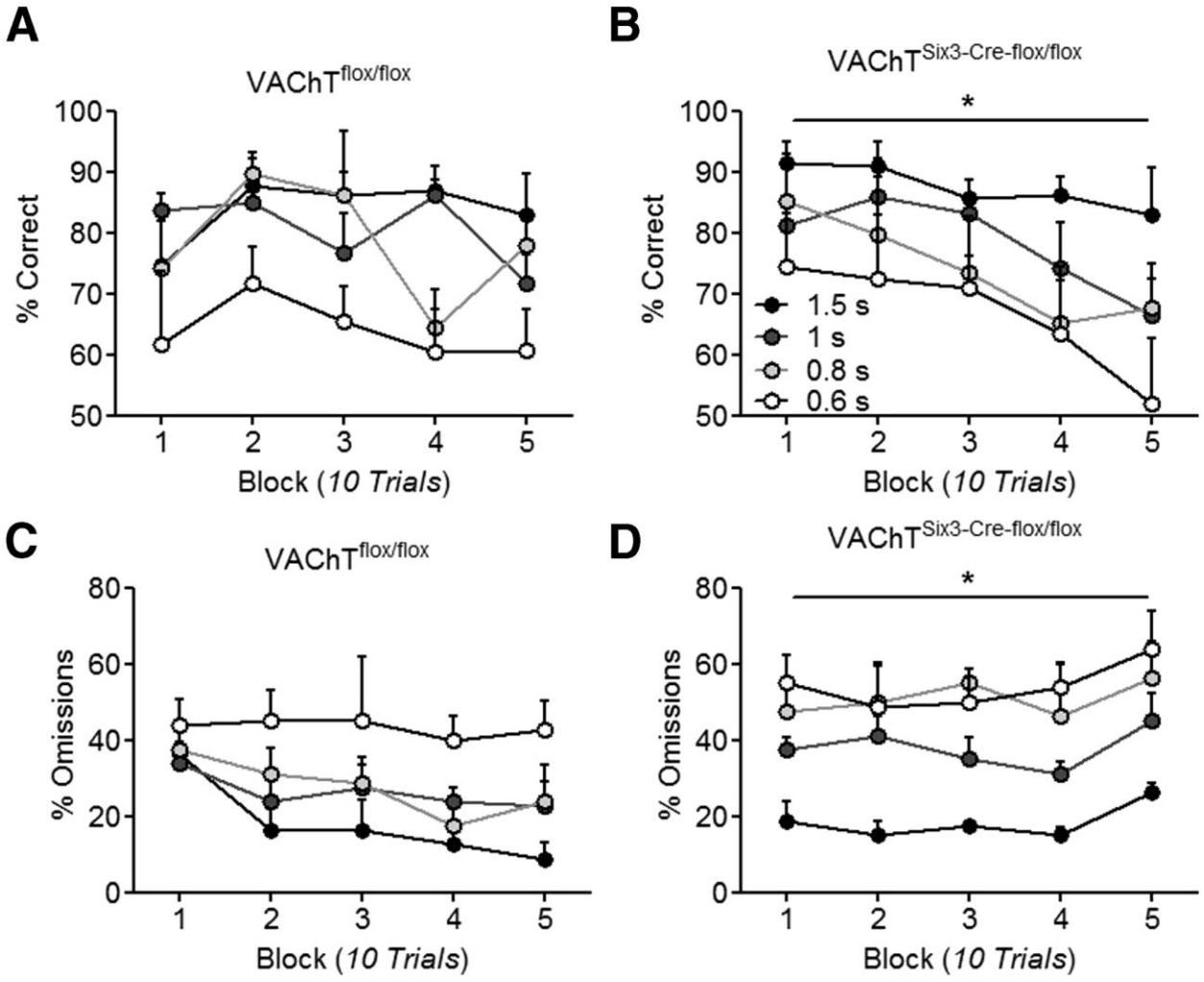


3.4.5 Deletion of forebrain VAChT impairs sustained attention

To assess sustained attention (vigilance), we analyzed both response accuracy and rate of omissions over blocks of 10 trials (Romberg et al., 2011). Response accuracy of control mice did not significantly vary across blocks, but did reduce significantly with decreases in stimuli duration (RM-ANOVA, main effect of stimulus duration $F(3,48) = 5.893$, $p = 0.0104$; no effect of block $F(4,48) = 1.214$, $p = 0.317$; Figure 3.6A). In contrast, response accuracy of VAChTSix3-Cre-flox/flox mice reduced significantly across blocks and stimuli duration (RM-ANOVA, main effect of stimulus duration $F(3,48) = 4.257$, $p = 0.0077$; main effect of block $F(4,48) = 3.933$, $p = 0.0289$; Figure 3.6B). As with response accuracy, rate of omissions of control mice did not vary across blocks, only with stimuli duration (RM-ANOVA, main effect of stimulus duration $F(3,48) = 5.803$, $p = 0.0228$; no effect of block $F(4,48) = 0.5352$, $p = 0.7105$; Figure 3.6C), whereas rate of omissions of VAChTSix3-Cre-flox/flox mice increased significantly across stimuli duration and blocks (RM-ANOVA, main effect of stimulus duration $F(3,48) = 9.387$, $p = 0.0018$; main effect of block $F(4,48) = 2.803$, $p = 0.0360$; Figure 3.6D). Together the data suggest that VAChTSix3-Cre-flox/flox mice have impaired ability to sustain attention.

Figure 3.6. VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice have impaired sustained attention. Mean response accuracy for blocks of 10 trials for (***A***) VAChT$^{flox/flox}$ and (***B***) VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice. Mean rate of omission for blocks of 10 trials for (***C***) VAChT$^{flox/flox}$ and (***D***) VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice (n = 6, data are mean ± SEM, *p < 0.05).

Figure 3.6. VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice have impaired sustained attention.

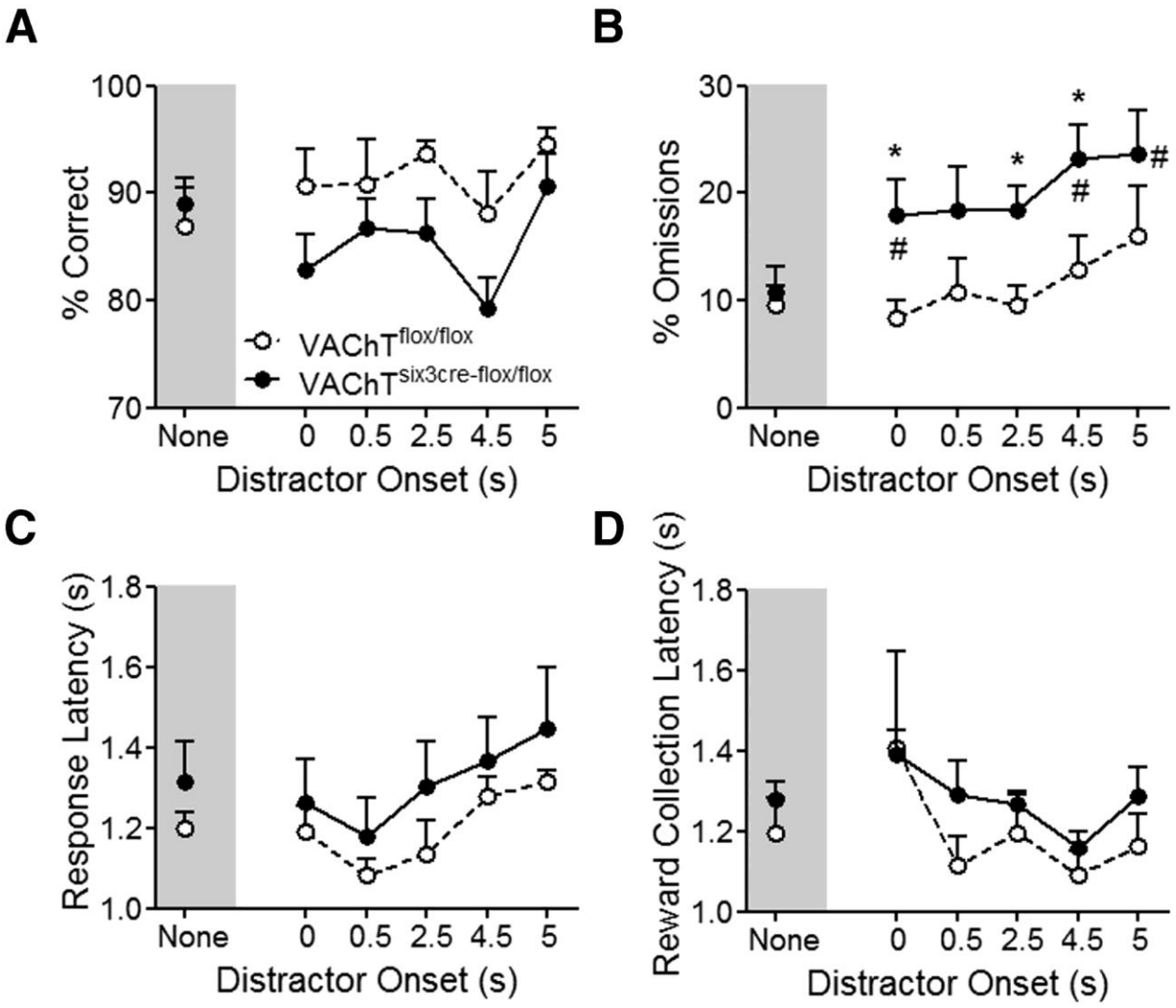


3.4.6 Deletion of forebrain VAChT increases susceptibility to distractions

To assess distractibility of VAChTSix3-Cre-flox/flox mice, we increased attentional demand by testing them on a distraction variation of the 5-CSRT. Stimulus duration was set to 2 s, where performance of both genotypes was identical, and distractor sounds were played at set time points during trials. Response accuracy of VAChTSix3-Cre-flox/flox mice tended to be reduced by distractions compared with controls (RM-ANOVA, near significant effect of genotype $F(1,32) = 4.825$, $p = 0.0589$; main effect of distractor onset $F(4,32) = 3.583$, $p = 0.0159$; Figure 3.7A). Rate of omission was significantly higher in VAChTSix3-Cre-flox/flox mice than controls (RM-ANOVA, significant effect of genotype $F(1,32) = 6.809$, $p = 0.0312$; no effect of distractor onset $F(4,32) = 2.564$, $p = 0.0572$; Figure 3.7B). Importantly, neither response latency (RM-ANOVA, no effect of genotype $F(1,32) = 1.045$, $p = 0.3367$; main effect of distractor onset $F(4,32) = 7.069$, $p = 0.0003$; Figure 3.7C) nor reward collection latency varied between genotypes (RM-ANOVA, no effect of genotype $F(1,32) = 0.7467$, $p = 0.4127$; main effect of distractor onset $F(4,32) = 3.226$, $p = 0.0248$; Figure 3.7D).

Figure 3.7 VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice are more susceptible to distraction. Mice were subjected to a series of distraction trials and the average values of 50 trial sessions per distractor onset are plotted.***A***, Mean response accuracy. ***B***, Mean rate of omission. ***C***, Mean response latency. ***D***, Mean reward collection latency (n = 6, data are mean ± SEM, * represents significant differences between genotypes, # represents significant differences within genotypes; *$p < 0.05$).

Figure 3.7 VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice are more susceptible to distraction.



3.4.7 Galantamine improves attention in wild-type mice on a demanding task

Detection of pro-attentive effects of pharmacological manipulations on the 5-CSRT is hampered by possible ceiling effects (Robbins, 2002). To detect differences in mice it is important to test the drug on challenging conditions, which can be achieved by shortening the duration of the stimulus presentation (Romberg et al., 2011). To find a suitably challenging task in which we could observe increased attention, we trained a group (n = 8) of wild-type mice on the 5-CSRT, and ran them through the probe trial series, serially reducing stimulus duration (1.5, 1, 0.8, and 0.6 s). Choice accuracy ($F(4,31) = 13.77$, $p < 0.0001$; Fig. 3.8A) and rate of omissions ($F(4,31) = 6.716$, $p = 0.0024$; Fig. 3.8B) were significantly affected by reducing stimulus duration. Post hoc analysis revealed that at 0.6 s stimulus duration the rate of accuracy was significantly reduced, and omissions significantly increased from the 1.5 s stimulus duration. We therefore chose both, 0.8 s stimulus duration and the more challenging 0.6 s stimulus duration, for pharmacological testing. Administration of galantamine (1 mg/kg, i.p.) 1 h before 5-CSRT testing at 0.8 s did not improve accuracy (paired t test, $t(7) = 1.287$, $p = 0.2544$; Fig. 3.8C) or omissions (paired t test, $t(7) = 0.4581$, $p = 0.6661$; Fig. 3.8D). However, when administered before a 0.6 s stimulus duration session, galantamine (1 mg/kg) significantly improved accuracy compared with saline (paired t test, $t(7) = 2.405$, $p = 0.0471$; Fig. 3.8E) and significantly reduced the rate of omission (paired t test, $t(7) = 2.379$, $p = 0.0489$;Fig. 3.8F). Response latency (paired t test, $t(7) = 1.296$, $p = 0.2360$), and reward collection latency (paired t test, $t(7) = 0.390$, $p = 0.7080$) were not changed by galantamine injections (data not shown). These results suggest that increased cholinergic tone can increase attentional performance when the probe trial is sufficiently demanding to avoid a potential ceiling effect.

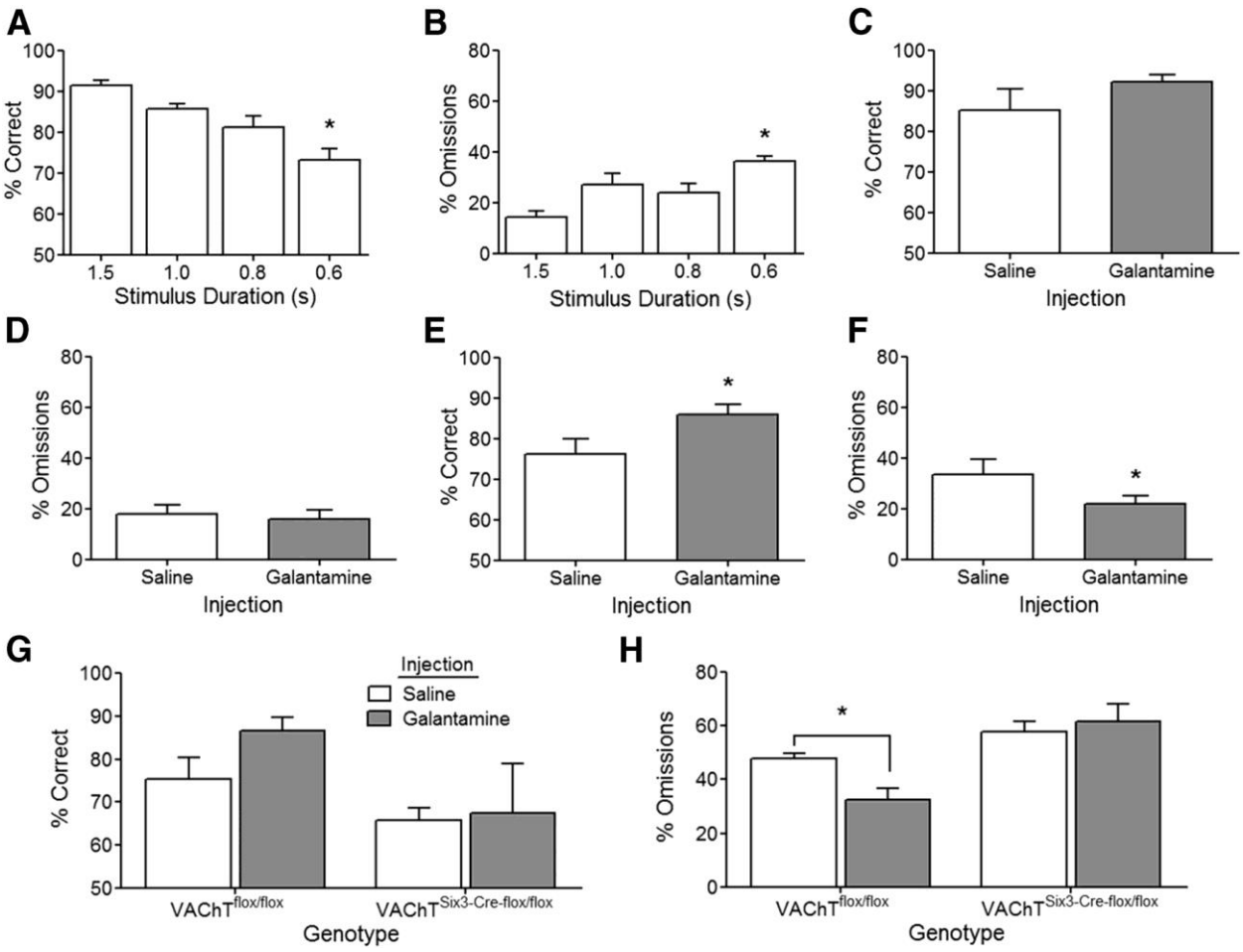
3.4.8 Galantamine does not improve attention deficits in VAChTSix3-Cre-flox/flox mice

To investigate if the deficits observed in VAChTSix3-Cre-flox/flox are exclusively related to decreased levels of synaptic ACh, we injected mice with galantamine (1 mg/Kg IP), a

dose that effectively improved performance of wild-type mice (Fig. 3.8), and tested both VAChTSix3-Cre-flox/flox and VAChTflox/flox mice at 0.6 s (50 trials). Galantamine tended to improve choice accuracy, albeit this effect did not reach statistical significance (paired t test, $t(5) = 1.954$, $p = 0.1224$; Fig. 3.8G). However, the drug was able to significantly reduce rate of omissions (paired t test, $t(5) = 3.383$, $p = 0.0277$; Fig. 3.8H) in VAChTflox/flox mice. Interestingly, galantamine had no effect on the performance of VAChTSix3-Cre-flox/flox mice, neither improving accuracy (paired t test, $t(5) = 0.162$, $p = 0.880$; Fig. 8G) nor rate of omission (paired t test, $t(5) = 0.868$, $p = 0.434$; Fig. 3.8H).

Figure 3.8 Galantamine improves attention in wild-type, but not in VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice. Wild-type mice (*n* = 8) were subjected to a series of probe trials and the average values of 50 trial sessions are plotted. ***A***, Mean accuracy. ***B***, Mean rate of omissions. Effect of galantamine (1 mg/kg i.p.) on (***C***) response accuracy and (***D***) rate of omission in wild-type mice at a 0.8 s stimulus duration (*n* = 8). Effect of galantamine on (***E***) response accuracy and (***F***) rate of omission in wild-type mice at a 0.6 s stimulus duration (*n* = 8). Effect of galantamine (1 mg/Kg I.P.) on (***G***) choice accuracy and (***H***) rate of omission in VAChT$^{flox/flox}$ and VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$mice (*n* = 6, data are mean ± SEM, **p* < 0.05).

Figure 3.8 Galantamine improves attention in wild-type, but not in VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice.

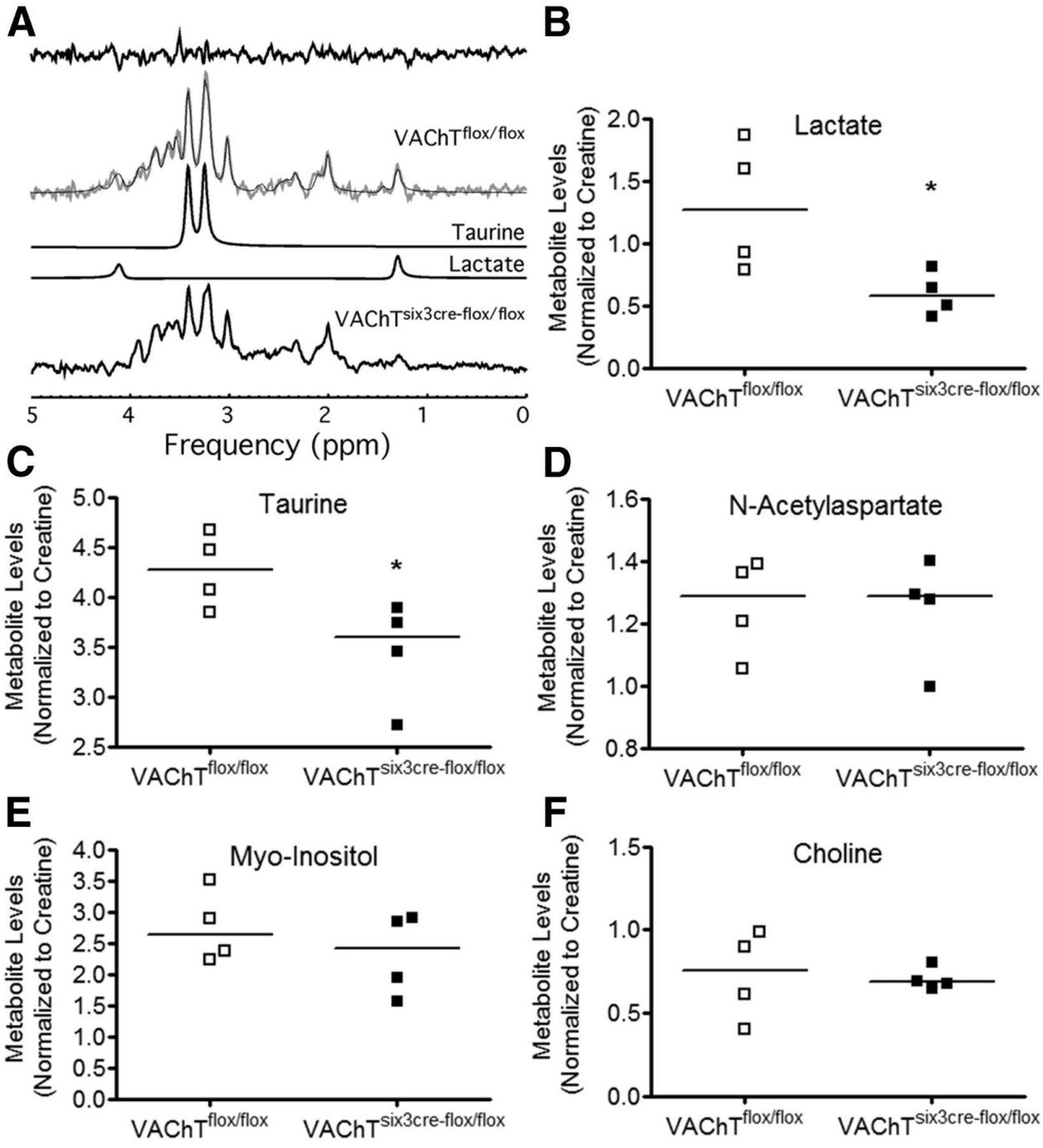


3.4.9 Deletion of forebrain VAChT results in metabolic abnormalities in the PFC

The lack of effect of galantamine in VAChTSix3-Cre-flox/flox suggests that diminished ACh release in mutant mice may not be sufficiently increased by galantamine to reverse the attentional deficits in these mice. In addition to that, a chronic decrease in cholinergic tone may cause circuitry or metabolic changes that could affect how neuronal circuitries are recruited for specific cognitive tasks. We used in vivo magnetic resonance spectroscopy to assess if metabolic parameters were affected in the prefrontal region of VAChT-deficient mice (Fig. 3.9A; representative spectra). This analysis revealed that VAChTSix3-Cre-flox/flox mice had significantly less Lac ($t(6) = 2.600$, $p = 0.0428$; Fig. 3.9B) and Tau ($t(6) = 2.522$, $p = 0.0452$; Fig. 3.9C) than controls. Levels of NAA ($t(6) = 0.0907$, $p = 0.9307$; Fig. 3.9D), Myo ($t(6) = 0.9598$, $p = 0.3742$; Fig. 3.9E), and Cho ($t(6) = 0.1461$, $p = 0.8886$; Fig. 3.9F) remained unchanged.

Figure 3.9. VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice have metabolic abnormalities in the PFC. *In vivo* magnetic resonance spectroscopy of neuronal metabolites. ***A***, 9.4 tesla ^{1}H magnetic resonance spectroscopy data from VAChT$^{flox/flox}$ (top) and VAhT$^{six3cre\text{-}flox/flox}$ (bottom) mice. The VAChT$^{flox/flox}$ data are shown in gray with fit superimposed (black line) and residual shown above. Individual component spectra for Tau and Lac are also provided. ***B***, Levels of Lac. ***C***, Levels of Tau. ***D***, Levels of NAA. ***E***, Levels of Myo. ***F***, Levels of Cho (n = 4, data shown are the median and individual values per mouse, *p < 0.05).

Figure 3.9. VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice have metabolic abnormalities in the PFC.



3.4.10 Deletion of forebrain VAChT results in altered RNA metabolism in the PFC

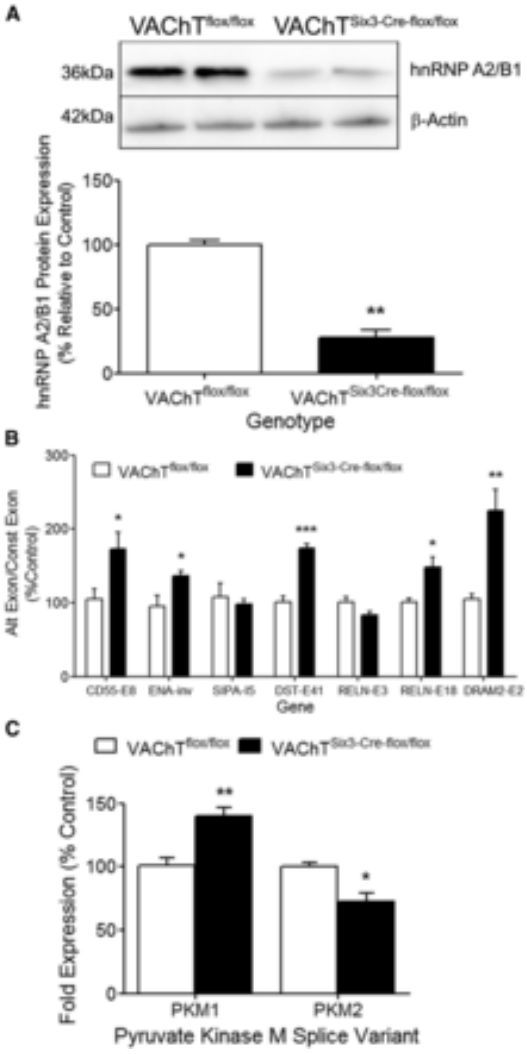
Cholinergic deficits in AD have been proposed to cause major transcriptome changes via aberrant hnRNPA2/B1 expression (Berson et al., 2012). hnRNPA2/B1 refers to a family of proteins that functions as splicing factors and mRNA chaperones (Hoek et al., 1998; Kamma et al., 1999). This gene family is critical for regulating alternative splicing in numerous genes involved with synaptic plasticity and cognition (Berson et al., 2012). Its expression is severely reduced in Alzheimer brain and appears enhanced in primary mouse neurons under carbachol induction (Berson et al., 2012), suggesting cholinergic regulation of hnRNPA2/B1 proteins. To determine whether the PFC of VAChTSix3-Cre-flox/flox shows parallel suppression of the hnRNPA2/B1 proteins, their levels were assessed. Compared with controls, the PFC of VAChTSix3-Cre-flox/flox mice showed a significant reduction in hnRNPA2/B1 expression (75% decrease, $t(6) = 4.941$, $p = 0.0026$; Fig. 3.10A). We then used qPCR to assess whether the decrease in hnRNPA2/B1 could have a functional impact on RNA processing in the PFC. We evaluated the alternative splicing of the key genes, SIPA1L1(SIPA), REELIN(RELN), DRAM2, CD55, DYSTONIN (DST), and ENAH, as manifested by increased exon inclusion tested both in Alzheimer's brain and in mouse brain depleted of its cholinergic neurons by saporin-mediated treatment (Berson et al., 2012). This analysis revealed that VAChTSix3-Cre-flox/flox mice had significant changes in the splicing of these genes in the PFC, with significantly increased inclusion of exon 8 of CD55 ($t(10) = 2.550$, $p = 0.0289$), exon 41 of DST ($t(10) = 7.436$, $p = 0.0001$), exon 18 of RELN ($t(10) = 3.230$, $p = 0.0090$), and exon 2 of DRAM2 ($t(10) = 3.990$, $p = 0.0260$). In addition the inv isoform of the ENAH gene was also significantly increased ($t(10) = 2.522$, $p = 0.0303$). In contrast, intron 5 of SIPA ($t(10) = 0.5449$, $p = 0.5978$) and exon 3 of RELN ($t(10) = 2.215$, $p = 0.0511$), which have also been shown to be affected in AD, were unchanged, although we detected a trend for the latter (Fig. 3.10B).

hnRNPA2/B1 has been shown to regulate the splicing of pyruvate kinase M (PKM) enzyme, which in turn dictates lactate metabolism (David et al., 2010). Knockdown of hnRNPA2/B1 expression in vitro favors the PKM1 isoform, which leads to reduced

levels of Lac, whereas upregulation hnRNPA2/B1 favors the PKM2 isoform and increased Lac levels (Clower et al., 2010; for review, see Chen et al., 2010). To test whether alternative splicing of PKM occurs in VAChTSix3-Cre-flox/flox mice, we performed qPCR to determine the expression of the PKM1 and PKM2 splice variants. Interestingly the VAChTSix3-Cre-flox/flox mice showed a significant upregulation of the PKM1 variant ($t(10) = 4.277$, $p = 0.0016$), and a significant reduction in the PKM2 variant ($t(10) = 3.073$, $p = 0.0110$; Fig. 3.10C), effectively changing the ratio between these two enzymes.

Figure 3.10. VAChTSix3-Cre-flox/flox mice have abnormal RNA processing in the PFC. A, hnRNPA2/B1 protein expression in the PFC with representative immunoblots (inset, n = 4). B, qPCR analysis of alternative splicing events for CD55, ENAH, SIPA, Dystonin (DST), Reelin (RELN), and DRAM2. C, Expression of the PKM1 and PKM2 isoforms. Alternative exon levels are normalized to a constitutive exon from the same gene (n = 6, data are mean ± SEM, *p < 0.05, **p < 0.01, and ***p < 0.001).

Figure 3.10. VAChTSix3-Cre-flox/flox mice have abnormal RNA processing in the PFC.



3.5 Discussion

In this report we show that forebrain VAChT knock-out mice present deficits in two domains of executive function, cognitive flexibility and attention. Additionally, VAChT-deficient mice exhibit metabolic deficits in the PFC suggestive of changes in PFC circuitry. Furthermore, these mice have reduced expression of a key splicing factor, hnRNPA2/B1, which has been previously implicated in synaptic plasticity deficits in AD (Berson et al., 2012) and in mutations that were recently discovered in rare proteinopathies (Kim et al., 2013). These changes have a functional impact on RNA metabolism in the PFC of VAChT-deficient mice. These results suggest that VAChTSix3-Cre-flox/flox mice may represent a powerful tool to dissect the molecular and neurochemical basis of executive dysfunction.

3.5.1 Cognitive flexibility in VAChTSix3-Cre-flox/flox mice

VAChTSix3-Cre-flox/flox mice were able to associate an image with a reward, and another with a punishment, in the pairwise visual discrimination task. Acquisition of this task has been shown to be dependent on glutamatergic signaling, with mice lacking the GLAST glutamate transporter being unable to acquire the task (Karlsson et al., 2009). Evidence suggests that the NMDA receptor is an important molecular switch for the acquisition of the task (Brigman et al., 2008; Barkus et al., 2012). Our results indicate that cholinergic signaling is not required for such learning. In contrast, when contingencies of the pairwise task were reversed, forebrain VAChT knock-out mice were unable to adapt and learn the new rule. Reversal learning in a visual discrimination task has been proposed to serve as a measure of cognitive flexibility in rodents (Izquierdo et al., 2006; Brigman et al., 2008). Neurochemical modulation of reversal learning is complex, as it can be enhanced by targeting multiple neurotransmitter signaling systems including serotonergic (Brigman et al., 2010), dopaminergic (Izquierdo et al., 2006), and glutamatergic (Balschun et al., 2010). The role of cholinergic signaling has been focused predominantly on muscarinic receptors (Ridley et al., 1984, 1985). Determining the exact mechanism has been difficult, as M1 receptor agonists facilitate cognitive flexibility (McCool et al., 2008; Shirey et al., 2009), but no

effects on cognitive flexibility were observed in M1-null mice (Bartko et al., 2011). There is evidence suggesting that M2 and M4 receptors may be involved in cognitive flexibility (Nieves-Martinez et al., 2012). Our results indicate that cholinergic signaling is essential for modulation of cognitive flexibility.

3.5.2 Attention deficits in VAChT-deficient mice

To further understand the role of forebrain ACh in executive function we evaluated attention on forebrain VAChT knock-out mice using the 5-CSRT task, a test suggested to be dependent on PFC cholinergic signaling (Guillem et al., 2011; for review, see Robbins, 2000; Dalley et al., 2004a). In line with our previous finding with the VAChTSix3-Cre-flox/flox mouse line having learning and memory deficits (Martyn et al., 2012), these mice showed impairments in acquisition of the 5-CSRT, taking nearly twice as long as controls to reach criteria at the 4 and 2 s phase of the training process. Interestingly, they were not impaired during the pretraining phase, wherein mice are taught to respond to the touchscreen. These results suggest that simple operant conditioning is not dependent on forebrain cholinergic signaling, but rather that forebrain ACh is responsible for the use of such information to perform higher order cognitive tasks. Attentional demand deficits were probed by reducing stimulus duration. Choice accuracy, perseverative, and premature responses of VAChTSix3-Cre-flox/flox mice were unaffected. However, the rate of omission for VAChTSix3-Cre-flox/flox was significantly increased. Additionally, in the presence of a noise distractor VAChTSix3-Cre-flox/flox mice were significantly less attentive than controls, showing a much higher rate of omissions. This test was performed under a condition in which the performance of VAChT-deficient mice was on par with controls without the auditory distraction. The auditory distraction task gives a clear indication that the attention deficits observed in VAChTSix3-Cre-flox/flox mice were not due to visual abnormalities. These findings are aligned with previous work, given that cue detection has been shown to involve transient rises in ACh release (Himmelheber et al., 2000; Parikh et al., 2007). Our experiments agree with previous work indicating a key role for cholinergic activity in improving response to distractors in attentional tasks (Gill et al., 2000; Terry et al., 2002;

Newman and McGaughy, 2008; Broussard et al., 2009; Howe et al., 2010; St Peters et al., 2011).

Regulation of attentional performance by endogenous ACh has been investigated in rats using immunolesion with IgG-192 saporin (Walsh et al., 1996; Risbrough et al., 2002; Lehmann et al., 2003; Chudasama et al., 2004; Dalley et al., 2004b). Specifically, deficits in choice accuracy and increases in perseverative responses were observed in rats following 192 IgG-saporin-induced lesions (McGaughy et al., 2002; Dalley et al., 2004b). In these studies no effects were observed on rates of omissions. Our experiments show a somewhat distinct feature, demonstrating increased rates of omission, without changes in perseverative response. Interestingly, selective elimination of the β2 nAChR in mice also revealed an increase in rate of omissions (Guillem et al., 2011). The differences between genetically modified and lesioned animals could therefore be species related. Alternatively, this difference may reflect the capacity of cholinergic neurons to secrete more than one neurotransmitter (El Mestikawy et al., 2011; Prado et al., 2013). Basal forebrain cholinergic neurons can release glutamate in vitro (Allen et al., 2006). There is also evidence that these neurons possess the machinery to release GABA (Henny and Jones, 2008). Recent experiments targeting striatum cholinergic neurons revealed striking behavioral differences between mice that had cholinergic elimination, using immunolesion, or mice that were genetically targeted to eliminate striatal ACh release (Kitabatake et al., 2003; Guzman et al., 2011; for review, see Prado et al., 2013). Immunolesion of cholinergic neurons can therefore have effects beyond impairing ACh release and could affect cotransmission. Whether cotransmission has a role in the small phenotypic differences between our experiments and previous work using rats injected with IgG-192-saporin remains to be determined.

Galantamine was unable to rescue the attention deficits in VAChTSix3-Cre-flox/flox mice. We have previously shown, using a mouse line with decreased VAChT expression, that galantamine could reverse social memory deficits (Prado et al., 2006) and object recognition memory deficits (de Castro et al., 2009b; De Jaeger et al., 2013). Hence, the fact that galantamine can improve performance of control mice, but not of VAChTSix3-Cre-flox/flox mice, suggests that the latter may not release sufficient

synaptic ACh to be enhanced by galantamine. Alternatively, these sophisticated tasks may be more affected by changes in neuronal circuitry. The in vivo 1H spectroscopy data suggest that critical metabolic changes occur in VAChT-deficient mice; namely, reduced levels of both Tau and Lac in the prefrontal region. Lac has been proposed as the preferred metabolic substrate for neurons (for review, see Pellerin et al., 2007), suggesting that decreased cholinergic tone may lead to a general decrease in neuronal activity.

3.5.3 Alterations in PFC function in VAChT-deficient mice

The reduction in PFC hnRNPA2/B1 observed in VAChTSix3-Cre-flox/flox mice is in line with previous work indicating cholinergic-mediated regulation of its expression in AD (Berson et al., 2012). Furthermore, we found that alternative splicing alteration pattern in the PFC of these mice is similar to the pattern found in both AD patient samples and hypocholinergic animal models (Berson et al., 2012). Overall, these changes suggest alterations in the PFC circuitry. Furthermore, the splicing change found in the PKM gene indicates a potential mechanism for cholinergic regulation of Lac metabolism. Of note, nearly significant differential expression of the PKM variants was observed in exon array datasets from the entorhinal cortices of three AD patients and three matched controls studied previously, despite the small number of samples (Berson et al., 2012; $p < 0.051$; raw data deposited in the GenBank).

Decreased Lac levels have been observed in mouse models of AD (Du Yan et al., 2000; Marjanska et al., 2005). Moreover, in the CRND8 transgenic mouse model of AD, lower levels of Tau were observed in vivo by H1-NMR (Salek et al., 2010). Interestingly, high levels of Lac in cultured neurons decrease susceptibility to Aβ-derived peptides and oxidative stress in vitro (Newington et al., 2012). Together, these data suggest that VAChTSix3-Cre-flox/flox mice may be useful for understanding metabolic abnormalities that occur in dementia.

3.6 Conclusion

In summary, by eliminating VAChT from the forebrain we determined that cholinergic signaling regulates executive function, affects metabolism, and also RNA processing in the PFC. The PFC has been shown to mediate salient cue detection (Himmelheber et al., 2000; Parikh et al., 2007), and it serves as a hub that regulates numerous neurotransmitter interactions (Dalley et al., 2004a; Carr et al., 2007; Tait and Brown, 2008). Our work helps to define the specific role played by ACh in behaviors related to cortical functioning, and its potential underlying mechanisms. Decreased levels of VAChT in the brain have been reported in AD (Efange et al., 1997; Chen et al., 2011), therefore the executive dysfunction and mRNA processing abnormalities we observed in VAChT-deficient mice may be of relevance to model this specific deficiency in humans. Therefore, this work provides novel insights into the basic neurochemical contributors governing executive function.

3.7 Acknowledgements

This work was supported by Canadian Institutes of Health Research (MOP 89919 and 126000, M.A.M.P. and V.F.P.), Natural Sciences and Engineering Research Council of Canada (V.F.P.), Canadian Foundation for Innovation, and Ontario Research Fund (M.A.M.P., V.F.P.).

3.8 References

Allen TG, Abogadie FC, Brown DA (2006) Simultaneous release of glutamate and acetylcholine from single magnocellular “cholinergic” basal forebrain neurons. J Neurosci 26:1588–1595.

Alvarez JA, Emory E (2006) Executive function and the frontal lobes: a meta-analytic review. Neuropsychol Rev 16:17–42.

Balschun D, Moechars D, Callaerts-Vegh Z, Vermaercke B, Van Acker N, Andries L, D'Hooge R (2010) Vesicular glutamate transporter VGLUT1 has a role in hippocampal long-term potentiation and spatial reversal learning. Cereb Cortex 20:684–693.

Barkus C, Feyder M, Graybeal C, Wright T, Wiedholz L, Izquierdo A, Kiselycznyk C, Schmitt W, Sanderson DJ, Rawlins JN, Saksida LM, Bussey TJ, Sprengel R, Bannerman D, Holmes A (2012) Do GluA1 knockout mice exhibit behavioral abnormalities relevant to the negative or cognitive symptoms of schizophrenia and schizoaffective disorder? Neuropharmacology 62:1263–1272.

Bartha R (2007) Effect of signal-to-noise ratio and spectral linewidth on metabolite quantification at 4 T. NMR Biomed 20:512–521.

Bartha R, Drost DJ, Williamson PC (1999) Factors affecting the quantification of short echo in-vivo 1H MR spectra: prior knowledge, peak elimination, and filtering. NMR Biomed 12:205–216.

Bartha R, Drost DJ, Menon RS, Williamson PC (2000a) Comparison of the quantification precision of human short echo time (1)H spectroscopy at 1.5 and 4.0 Tesla. Magn Reson Med 44:185–192.

Bartha R, Drost DJ, Menon RS, Williamson PC (2000b) Spectroscopic lineshape correction by QUECC: combined QUALITY deconvolution and eddy current correction. Magn Reson Med 44:641–645.

Bartha R, Smith M, Rupsingh R, Rylett J, Wells JL, Borrie MJ (2008) High field (1)H MRS of the hippocampus after donepezil treatment in Alzheimer disease. Prog Neuropsychopharmacol Biol Psychiatry 32:786–793.

Bartko SJ, Romberg C, White B, Wess J, Bussey TJ, Saksida LM (2011) Intact attentional processing but abnormal responding in M1 muscarinic receptor-deficient mice using an automated touchscreen method. Neuropharmacology 61:1366–1378.

Berson A, Barbash S, Shaltiel G, Goll Y, Hanin G, Greenberg DS, Ketzef M, Becker AJ, Friedman A, Soreq H (2012) Cholinergic-associated loss of hnRNP-A/B in Alzheimer's disease impairs cortical splicing and cognitive function in mice. EMBO Mol Med 4:730–742.

Brigman JL, Feyder M, Saksida LM, Bussey TJ, Mishina M, Holmes A (2008) Impaired discrimination learning in mice lacking the NMDA receptor NR2A subunit. Learn Mem 15:50–54.

Brigman JL, Mathur P, Harvey-White J, Izquierdo A, Saksida LM, Bussey TJ, Fox S, Deneris E, Murphy DL, Holmes A (2010) Pharmacological or genetic inactivation of the serotonin transporter improves reversal learning in mice. Cereb Cortex 20:1955–1963.

Broussard JI, Karelina K, Sarter M, Givens B (2009) Cholinergic optimization of cue-evoked parietal activity during challenged attentional performance. Eur J Neurosci 29:1711–1722.

Carr DB, Andrews GD, Glen WB, Lavin A (2007) alpha2-Noradrenergic receptors activation enhances excitability and synaptic integration in rat prefrontal cortex pyramidal neurons via inhibition of HCN currents. J Physiol 584:437–450.

Chen GJ, Xiong Z, Yan Z (2013) Abeta impairs nicotinic regulation of inhibitory synaptic transmission and interneuron excitability in prefrontal cortex. Mol Neurodegener 8:3.

Chen KH, Reese EA, Kim HW, Rapoport SI, Rao JS (2011) Disturbed neurotransmitter transporter expression in Alzheimer's disease brain. J Alzheimers Dis 26:755–766. MedlineGoogle Scholar

Chen M, Zhang J, Manley JL (2010) Turning on a fuel switch of cancer: hnRNP proteins regulate alternative splicing of pyruvate kinase mRNA. Cancer Res 70:8977–8980.

Chudasama Y (2011) Animal models of prefrontal-executive function. Behav Neurosci 125:327–343.

Chudasama Y, Dalley JW, Nathwani F, Bouger P, Robbins TW (2004) Cholinergic modulation of visual attention and working memory: dissociable effects of basal forebrain 192-IgG-saporin lesions and intraprefrontal infusions of scopolamine. Learn Mem 11:78–86.

Clower CV, Chatterjee D, Wang Z, Cantley LC, Vander Heiden MG, Krainer AR (2010) The alternative splicing repressors hnRNP A1/A2 and PTB influence pyruvate kinase isoform expression and cell metabolism. Proc Natl Acad Sci U S A 107:1894–1899.

Dalley JW, Cardinal RN, Robbins TW (2004a) Prefrontal executive and cognitive functions in rodents: neural and neurochemical substrates. Neurosci Biobehav Rev 28:771–784.

Dalley JW, Theobald DE, Bouger P, Chudasama Y, Cardinal RN, Robbins TW (2004b) Cortical cholinergic function and deficits in visual attentional performance in rats following 192 IgG-saporin-induced lesions of the medial prefrontal cortex. Cereb Cortex 14:922–932.

David CJ, Chen M, Assanah M, Canoll P, Manley JL (2010) HnRNP proteins controlled by c-Myc deregulate pyruvate kinase mRNA splicing in cancer. Nature 463:364–368.

de Castro BM, De Jaeger X, Martins-Silva C, Lima RD, Amaral E, Menezes C, Lima P, Neves CM, Pires RG, Gould TW, Welch I, Kushmerick C, Guatimosim C, Izquierdo I, Cammarota M, Rylett RJ, Gomez MV, Caron MG, Oppenheim RW, Prado MA, et al. (2009a) The vesicular acetylcholine transporter is required for neuromuscular development and function. Mol Cell Biol 29:5238–5250.

de Castro BM, Pereira GS, Magalhães V, Rossato JI, De Jaeger X, Martins-Silva C, Leles B, Lima P, Gomez MV, Gainetdinov RR, Caron MG, Izquierdo I, Cammarota M,

Prado VF, Prado MA (2009b) Reduced expression of the vesicular acetylcholine transporter causes learning deficits in mice. Genes Brain Behav 8:23–35.

De Jaeger X, Cammarota M, Prado MA, Izquierdo I, Prado VF, Pereira GS (2013) Decreased acetylcholine release delays the consolidation of object recognition memory. Behav Brain Res 238:62–68.

DelaBarre L, Garwood M (1998) Proceedings of the International Society of Magnetic Resonance in Medicine LASER: adiabatic single shot localization with J-refocUsing, p 358. Google Scholar

Du Yan S, Zhu Y, Stern ED, Hwang YC, Hori O, Ogawa S, Frosch MP, Connolly ES Jr., McTaggert R, Pinsky DJ, Clarke S, Stern DM, Ramasamy R (2000) Amyloid beta-peptide-binding alcohol dehydrogenase is a component of the cellular response to nutritional stress. J Biol Chem 275:27100–27109.

Efange SM, Garland EM, Staley JK, Khare AB, Mash DC (1997) Vesicular acetylcholine transporter density and Alzheimer's disease. Neurobiol Aging 18:407–413.

Elliott R (2003) Executive functions and their disorders: imaging in clinical neuroscience. Br Med Bull 65:49–59.

El Mestikawy S, Wallén-Mackenzie A, Fortin GM, Descarries L, Trudeau LE (2011) From glutamate co-release to vesicular synergy: vesicular glutamate transporters. Nat Rev Neurosci 12:204–216.

Evers EA, van der Veen FM, Fekkes D, Jolles J (2007) Serotonin and cognitive flexibility: neuroimaging studies into the effect of acute tryptophan depletion in healthy volunteers. Curr Med Chem 14:2989–2995.

Gill TM, Sarter M, Givens B (2000) Sustained visual attention performance-associated prefrontal neuronal activity: evidence for cholinergic modulation. J Neurosci 20:4745–4757.

Graybeal C, Feyder M, Schulman E, Saksida LM, Bussey TJ, Brigman JL, Holmes A (2011) Paradoxical reversal learning enhancement by stress or prefrontal cortical damage: rescue with BDNF. Nat Neurosci 14:1507–1509.

Grottick AJ, Higgins GA (2000) Effect of subtype selective nicotinic compounds on attention as assessed by the five-choice serial reaction time task. Behav Brain Res 117:197–208.

Guillem K, Bloem B, Poorthuis RB, Loos M, Smit AB, Maskos U, Spijker S, Mansvelder HD (2011) Nicotinic acetylcholine receptor beta2 subunits in the medial prefrontal cortex control attention. Science 333:888–891.

Guzman MS, De Jaeger X, Raulic S, Souza IA, Li AX, Schmid S, Menon RS, Gainetdinov RR, Caron MG, Bartha R, Prado VF, Prado MA (2011) Elimination of the vesicular acetylcholine transporter in the striatum reveals regulation of behaviour by cholinergic-glutamatergic co-transmission. PLoS Biol 9:e1001194.

Hasselmo ME, Sarter M (2011) Modes and models of forebrain cholinergic neuromodulation of cognition. Neuropsychopharmacology 36:52–73.

Henny P, Jones BE (2008) Projections from basal forebrain to prefrontal cortex comprise cholinergic, GABAergic and glutamatergic inputs to pyramidal cells or interneurons. Eur J Neurosci 27:654–670.

Hill EL (2004) Executive dysfunction in autism. Trends Cogn Sci 8:26–32.

Himmelheber AM, Sarter M, Bruno JP (2000) Increases in cortical acetylcholine release during sustained attention performance in rats. Brain Res Cogn Brain Res 9:313–325.

Hoek KS, Kidd GJ, Carson JH, Smith R (1998) hnRNP A2 selectively binds the cytoplasmic transport sequence of myelin basic protein mRNA. Biochemistry 37:7021–7029.

Howe WM, Ji J, Parikh V, Williams S, Mocaër E, Trocmé-Thibierge C, Sarter M (2010) Enhancement of attentional performance by selective stimulation of alpha4beta2(*) nAChRs: underlying cholinergic mechanisms. Neuropsychopharmacology 35:1391–1401.

Izquierdo A, Wiedholz LM, Millstein RA, Yang RJ, Bussey TJ, Saksida LM, Holmes A (2006) Genetic and dopaminergic modulation of reversal learning in a touchscreen-based operant procedure for mice. Behav Brain Res 171:181–188.

Jurado MB, Rosselli M (2007) The elusive nature of executive functions: a review of our current understanding. Neuropsychol Rev 17:213–233.

Kamma H, Horiguchi H, Wan L, Matsui M, Fujiwara M, Fujimoto M, Yazawa T, Dreyfuss G (1999) Molecular characterization of the hnRNP A2/B1 proteins: tissue-specific expression and novel isoforms. Exp Cell Res 246:399–411.

Karlsson RM, Tanaka K, Saksida LM, Bussey TJ, Heilig M, Holmes A (2009) Assessment of glutamate transporter GLAST (EAAT1)-deficient mice for phenotypes relevant to the negative and executive/cognitive symptoms of schizophrenia. Neuropsychopharmacology 34:1578–1589.

Kassem MN, Bartha R (2003) Quantitative proton short-echo-time LASER spectroscopy of normal human white matter and hippocampus at 4 tesla incorporating macromolecule subtraction. Magn Reson Med 49:918–927.

Kim HJ, Kim NC, Wang YD, Scarborough EA, Moore J, Diaz Z, MacLea KS, Freibaum B, Li S, Molliex A, Kanagaraj AP, Carter R, Boylan KB, Wojtas AM, Rademakers R, Pinkus JL, Greenberg SA, Trojanowski JQ, Traynor BJ, Smith BN, et al. (2013) Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS. Nature 495:467–473.

Kitabatake Y, Hikida T, Watanabe D, Pastan I, Nakanishi S (2003) Impairment of reward-related learning by cholinergic cell ablation in the striatum. Proc Natl Acad Sci U S A 100:7965–7970.

Kolisnyk B, Guzman MS, Raulic S, Fan J, Magalhães AC, Feng G, Gros R, Prado VF, Prado MA (2013) ChAT-ChR2-EYFP mice have enhanced motor endurance but show deficits in attention and several additional cognitive domains. J Neurosci 33:10427–10438.

Lehmann O, Grottick AJ, Cassel JC, Higgins GA (2003) A double dissociation between serial reaction time and radial maze performance in rats subjected to 192 IgG-saporin lesions of the nucleus basalis and/or the septal region. Eur J Neurosci 18:651–666.

Marjanska M, Curran GL, Wengenack TM, Henry PG, Bliss RL, Poduslo JF, Jack CR Jr., Ugurbil K, Garwood M (2005) Monitoring disease progression in transgenic mouse models of Alzheimer's disease with proton magnetic resonance spectroscopy. Proc Natl Acad Sci U S A 102:11906–11910.

Martins-Silva C, De Jaeger X, Guzman MS, Lima RD, Santos MS, Kushmerick C, Gomez MV, Caron MG, Prado MA, Prado VF (2011) Novel strains of mice deficient for the vesicular acetylcholine transporter: insights on transcriptional regulation and control of locomotor behavior. PLoS One 6:e17611.

Martyn AC, De Jaeger X, Magalhães AC, Kesarwani R, Gonçalves DF, Raulic S, Guzman MS, Jackson MF, Izquierdo I, Macdonald JF, Prado MA, Prado VF (2012) Elimination of the vesicular acetylcholine transporter in the forebrain causes hyperactivity and deficits in spatial memory and long-term potentiation. Proc Natl Acad Sci U S A 109:17651–17656.

McCool MF, Patel S, Talati R, Ragozzino ME (2008) Differential involvement of M1-type and M4-type muscarinic cholinergic receptors in the dorsomedial striatum in task switching. Neurobiol Learn Mem 89:114–124.

McGaughy J, Decker MW, Sarter M (1999) Enhancement of sustained attention performance by the nicotinic acetylcholine receptor agonist ABT-418 in intact but not basal forebrain-lesioned rats. Psychopharmacology 144:175–182.

McGaughy J, Dalley JW, Morrison CH, Everitt BJ, Robbins TW (2002) Selective behavioral and neurochemical effects of cholinergic lesions produced by intrabasalis infusions of 192 IgG-saporin on attentional performance in a five-choice serial reaction time task. J Neurosci 22:1905–1913.

McGuinness B, Barrett SL, Craig D, Lawson J, Passmore AP (2010) Executive functioning in Alzheimer's disease and vascular dementia. Int J Geriatr Psychiatry 25:562–568. MedlineGoogle Scholar

Morice R (1990) Cognitive inflexibility and pre-frontal dysfunction in schizophrenia and mania. Br J Psychiatry 157:50–54.

Newington JT, Rappon T, Albers S, Wong DY, Rylett RJ, Cumming RC (2012) Overexpression of pyruvate dehydrogenase kinase 1 and lactate dehydrogenase A in nerve cells confers resistance to amyloid beta and other toxins by decreasing mitochondrial respiration and reactive oxygen species production. J Biol Chem 287:37245–37258.

Newman LA, McGaughy J (2008) Cholinergic deafferentation of prefrontal cortex increases sensitivity to cross-modal distractors during a sustained attention task. J Neurosci 28:2642–2650.

Nieves-Martinez E, Haynes K, Childers SR, Sonntag WE, Nicolle MM (2012) Muscarinic receptor/G-protein coupling is reduced in the dorsomedial striatum of cognitively impaired aged rats. Behav Brain Res 227:258–264.

Parikh V, Ji J, Decker MW, Sarter M (2010) Prefrontal beta2 subunit-containing and alpha7 nicotinic acetylcholine receptors differentially control glutamatergic and cholinergic signaling. J Neurosci 30:3518–3530.

Parikh V, Kozak R, Martinez V, Sarter M (2007) Prefrontal acetylcholine release controls cue detection on multiple timescales. Neuron 56:141–154.

Pellerin L, Bouzier-Sore AK, Aubert A, Serres S, Merle M, Costalat R, Magistretti PJ (2007) Activity-dependent regulation of energy metabolism by astrocytes: an update. Glia 55:1251–1262.

Perry EK, Gibson PH, Blessed G, Perry RH, Tomlinson BE (1977) Neurotransmitter enzyme abnormalities in senile dementia. Choline acetyltransferase and glutamic acid decarboxylase activities in necropsy brain tissue. J Neurol Sci 34:247–265.

Perry RJ, Hodges JR (1999) Attention and executive deficits in Alzheimer's disease: a critical review. Brain 122:383–404.

Pfeuffer J, Tkác I, Provencher SW, Gruetter R (1999) Toward an in vivo neurochemical profile: quantification of 18 metabolites in short-echo-time (1)H NMR spectra of the rat brain. J Magn Reson 141:104–120.

Prado VF, Martins-Silva C, de Castro BM, Lima RF, Barros DM, Amaral E, Ramsey AJ, Sotnikova TD, Ramirez MR, Kim HG, Rossato JI, Koenen J, Quan H, Cota VR, Moraes MF, Gomez MV, Guatimosim C, Wetsel WC, Kushmerick C, Pereira GS, et al. (2006) Mice deficient for the vesicular acetylcholine transporter are myasthenic and have deficits in object and social recognition. Neuron 51:601–612.

Prado VF, Roy A, Kolisnyk B, Gros R, Prado MA (2013) Regulation of cholinergic activity by the vesicular acetylcholine transporter. Biochem J 450:265–274.

Ridley RM, Barratt NG, Baker HF (1984) Cholinergic learning deficits in the marmoset produced by scopolamine and ICV hemicholinium. Psychopharmacology 83:340–345.

Ridley RM, Baker HF, Drewett B, Johnson JA (1985) Effects of ibotenic acid lesions of the basal forebrain on serial reversal learning in marmosets. Psychopharmacology 86:438–443.

Risbrough V, Bontempi B, Menzaghi F (2002) Selective immunolesioning of the basal forebrain cholinergic neurons in rats: effect on attention using the 5-choice serial reaction time task. Psychopharmacology 164:71–81.

Robbins TW (2000) From arousal to cognition: the integrative position of the prefrontal cortex. Prog Brain Res 126:469–483.

Robbins TW (2002) The 5-choice serial reaction time task: behavioural pharmacology and functional neurochemistry. Psychopharmacology 163:362–380.

Robbins TW, Roberts AC (2007) Differential regulation of fronto-executive function by the monoamines and acetylcholine. Cereb Cortex 17(Suppl 1):i151–160.

Romberg C, Horner AE, Bussey TJ, Saksida LM (2013) A touch screen-automated cognitive test battery reveals impaired attention, memory abnormalities, and increased response inhibition in the TgCRND8 mouse model of Alzheimer's disease. Neurobiol Aging 34:731–744.

Romberg C, Mattson MP, Mughal MR, Bussey TJ, Saksida LM (2011) Impaired attention in the 3x TgAD mouse model of Alzheimer's disease: rescue by donepezil (Aricept) J Neurosci 31:3500–3507.

Sala M, Braida D, Lentini D, Busnelli M, Bulgheroni E, Capurro V, Finardi A, Donzelli A, Pattini L, Rubino T, Parolaro D, Nishimori K, Parenti M, Chini B (2011) Pharmacologic rescue of impaired cognitive flexibility, social deficits, increased aggression, and seizure susceptibility in oxytocin receptor null mice: a neurobehavioral model of autism. Biol Psychiatry 69:875–882.

Salek RM, Xia J, Innes A, Sweatman BC, Adalbert R, Randle S, McGowan E, Emson PC, Griffin JL (2010) A metabolomic study of the CRND8 transgenic mouse model of Alzheimer's disease. Neurochem Int 56:937–947.

Sarter M, Givens B, Bruno JP (2001) The cognitive neuroscience of sustained attention: where top-down meets bottom-up. Brain Res Brain Res Rev 35:146–160.

Schmitt JA, Wingen M, Ramaekers JG, Evers EA, Riedel WJ (2006) Serotonin and human cognitive performance. Curr Pharm Des 12:2473–2486.

Shirey JK, Brady AE, Jones PJ, Davis AA, Bridges TM, Kennedy JP, Jadhav SB, Menon UN, Xiang Z, Watson ML, Christian EP, Doherty JJ, Quirk MC, Snyder DH, Lah JJ, Levey AI, Nicolle MM, Lindsley CW, Conn PJ (2009) A selective allosteric potentiator of the M1 muscarinic acetylcholine receptor increases activity of medial prefrontal cortical neurons and restores impairments in reversal learning. J Neurosci 29:14271–14286.

Stalnaker TA, Takahashi Y, Roesch MR, Schoenbaum G (2009) Neural substrates of cognitive inflexibility after chronic cocaine exposure. Neuropharmacology 56(Supplement 1):63–72.

St Peters M, Demeter E, Lustig C, Bruno JP, Sarter M (2011) Enhanced control of attention by stimulating mesolimbic-corticopetal cholinergic circuitry. J Neurosci 31:9760–9771.

Tait DS, Brown VJ (2008) Lesions of the basal forebrain impair reversal learning but not shifting of attentional set in rats. Behav Brain Res 187:100–108.

Terry AV Jr., Risbrough VB, Buccafusco JJ, Menzaghi F (2002) Effects of (+/−)-4-[[2-(1-methyl-2-pyrrolidinyl)ethyl]thio]phenol hydrochloride (SIB-1553A), a selective ligand for

nicotinic acetylcholine receptors, in tests of visual attention and distractibility in rats and monkeys. J Pharmacol Exp Ther 301:284–292.

Traykov L, Raoux N, Latour F, Gallo L, Hanon O, Baudic S, Bayle C, Wenisch E, Remy P, Rigaud AS (2007) Executive functions deficit in mild cognitive impairment. Cogn Behav Neurol 20:219–224.

Walsh TJ, Herzog CD, Gandhi C, Stackman RW, Wiley RG (1996) Injection of IgG 192-saporin into the medial septum produces cholinergic hypofunction and dose-dependent working memory deficits. Brain Res 726:69–79.

Whitehouse PJ, Price DL, Clark AW, Coyle JT, DeLong MR (1981) Alzheimer disease: evidence for selective loss of cholinergic neurons in the nucleus basalis. Ann Neurol 10:122–126.

Whitehouse PJ, Price DL, Struble RG, Clark AW, Coyle JT, Delon MR (1982) Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. Science 215:1237–1239.

Chapter 4

α7 nicotinic ACh receptor-deficient mice exhibit sustained attention impairments that are reversed by β2 nicotinic ACh receptor activation

4.1 Copyright Permission

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS

May 17, 2016

This Agreement between Benjamin Kolisnyk ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number	3871420739413
License date	May 17, 2016
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	British Journal of Pharmacology
Licensed Content Title	α7 nicotinic ACh receptor-deficient mice exhibit sustained attention impairments that are reversed by β2 nicotinic ACh receptor activation
Licensed Content Author	Benjamin Kolisnyk,Mohammed A. Al-Onaizi,Vania F. Prado,Marco A. M. Prado
Licensed Content Date	Sep 22, 2015
Pages	13
Type of use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print and electronic
Portion	Full article
Will you be translating?	No
Title of your thesis / dissertation	CHOLINERGIC MECHANISMS REGULATING COGNTIVE FUNCTION AND RNA METABOLISM
Expected completion date	Aug 2016
Expected size (number of pages)	300
Requestor Location	Benjamin Kolisnyk 1151 Richmond London, ON N6A 3K7 Canada Attn: Benjamin Kolisnyk
Billing Type	Invoice
Billing Address	Benjamin Kolisnyk 1151 Richmond London, ON N6A 3K7 Canada Attn: Benjamin Kolisnyk
Total	0.00 USD

Terms and Conditions

TERMS AND CONDITIONS

and the right to reproduce the Wiley Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Wiley Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto

- NEITHER WILEY NOR ITS LICENSORS MAKES ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND ITS LICENSORS AND WAIVED BY YOU.

- WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.

- You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.

- IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

- Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.

- The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition

of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.

- This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.
- Any fee required for this permission shall be non-refundable after thirty (30) days from receipt by the CCC.
- These terms and conditions together with CCC's Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.
- In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.
- WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
- This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.
- This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regards to such state's conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of process by registered or certified mail, return receipt requested, at the last known address of such party.

WILEY OPEN ACCESS TERMS AND CONDITIONS

Wiley Publishes Open Access Articles in fully Open Access Journals and in Subscription journals offering Online Open. Although most of the fully Open Access journals publish open access articles under the terms of the Creative Commons Attribution (CC BY) License only, the subscription journals and a few of the Open Access Journals offer a choice of Creative Commons Licenses. The license type is clearly identified on the article.

The Creative Commons Attribution License

The Creative Commons Attribution License (CC-BY) allows users to copy, distribute and transmit an article, adapt the article and make commercial use of the article. The CC-BY

4.2 Chapter Summary

Disruptions of executive function, including attentional deficits, are a hallmark of a number of diseases. ACh in the prefrontal cortex regulates attentive behaviour; however, the role of α7 nicotinic ACh receptor (α7nAChR) in attention is contentious. In order to probe attention, we trained both wild-type and α7nAChR knockout mice on a touch screen-based five-choice serial reaction time task (5-CSRT). Following training procedures, we then tested sustained attention using a probe trial experiment. To further differentiate the role of specific nicotinic receptors in attention, we then tested the effects of both α7nAChR and β2nAChR agonists on the performance of both wild-type and knockout mice on the 5-CSRT task. At low doses, α7nAChR agonists improved attentional performance of wild-type mice, while high doses had deleterious effects on attention. α7nAChR knockout mice displayed deficits in sustained attention that were not ameliorated by α7nAChR agonists. However, these deficits were completely reversed by the administration of a β2nAChR agonist. Furthermore, administration of a β2nAChR agonist in α7nAChR knockout mice elicited similar biochemical response in the prefrontal cortex as the administration of α7nAChR agonists in wild-type mice. Our experiments reveal an intricate relationship between distinct nicotinic receptors to regulate attentional performance and provide the basis for targeting β2nAChRs pharmacologically to decrease attentional deficits due to a dysfunction in α7nAChRs.

4.3 Introduction

Attentional performance can be severely compromised in different neuropsychiatric and neurodegenerative diseases, including schizophrenia and Alzheimer's disease (Mega and Cummings, 1994; Buckner, 2004). ACh release in the prefrontal cortex (PFC), a brain area known to play a central role in attention (Kastner and Ungerleider, 2000; Corbetta and Shulman, 2002; Buschman and Miller, 2007), is important for the regulation of attentive behaviour (Elliott, 2003). Schizophrenic patients present severe physiological and molecular dysfunctions in the PFC (Weinberger et al., 1986; Mirnics et al., 2000; Guillozet-Bongaarts et al., 2014). One of the more profound molecular changes is the loss of the α7 nicotinic ACh receptor (α7nAChR), encoded by the

CHRNA7 gene. Although the genetic linkage between the CHRNA7 gene and schizophrenia is complex, with studies pointing towards and against CHRNA7 as a risk gene for the disease (Xu et al., 2001; Zammit et al., 2007), robust decreases in protein and mRNA expression of the α7nAChR have been shown in the PFC of patients with schizophrenia (Guan et al., 1999; Guillozet-Bongaarts et al., 2014). Moreover, in Alzheimer's disease, Aβ peptides can bind to α7nAChRs (Wang et al., 2000) and disrupt their function (Chen et al., 2006).

There is accumulating evidence demonstrating that cue detection during attentional efforts is mediated by nicotinic receptor signalling (McGaughy et al., 1999a; Grottick and Higgins, 2000; Parikh et al., 2007, 2010). β2nAChRs are both necessary and sufficient to regulate attention in mice using a non-demanding five-choice serial reaction time task (5-CSRT) paradigm (Guillem et al., 2011). On the other hand, the role of α7nAChRs in attention is still not completely understood. Initial studies suggest that CHRNA7−/− mice present deficits in sustained attention (Hoyle et al., 2006; Young et al., 2007). However, it has been reported that for less demanding tasks no deficits were observed in α7nAChR-null mice (Guillem et al., 2011). Taken together, these results suggest that attentional deficits in CHRNA7−/− mice may depend on the attentional load. Pharmacological manipulations of α7nAChRs have also produced conflicting results, most likely because of the poor selectivity of the drugs used (Grottick and Higgins, 2000; Pichat et al., 2007; Rezvani et al., 2009; Wallace et al., 2011a). Interestingly, studies in humans have shown that agonists for the α7nAChR can improve the performance of patients suffering from schizophrenia on neurocognitive tests (Olincy et al., 2006; Olincy and Stevens, 2007).

Here, we report that α7nAChR-null mice present deficits in their ability to sustain attention in a demanding paradigm. Moreover, we found that activation of α7nAChRs increased biochemical signalling and attention in wild-type (WT) mice, but not in CHRNA7−/− mice. Interestingly, activation of β2nAChRs triggered similar biochemical pathways as α7nAChR agonists and reversed attentional deficits in α7nAChR-null mice. These results suggest that α7nAChRs may contribute to attention performance, but activation of β2nAChRs can bypass the deficits triggered by deficient α7nAChR

signalling. Our results suggest that the α7nAChR plays a role in sustained attention during demanding tasks and that β2nAChR drugs may be of potential use for correcting cognitive and molecular signalling deficits seen in psychiatric or neurological disorders in which α7nAChRs are affected.

4.4 Material and Methods

4.4.1 Animals

CHRNA7−/− mice (B6.129S7 nAChR Chrna7tm1Bay/J) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were housed in groups of three or four per cage in a temperature-controlled room with 12/12 h light/dark cycle (07:00–19:00 h), and water was provided ad libitum. Only male mice were used in these studies. For the 5-CSRT studies, mice were housed in pairs and restricted to 85% of their free-feed weight and maintained on 85% of their weight for the duration of the studies as described (Kolisnyk et al., 2013a,b). All behavioural experiments were conducted between 12:00 and 17:00 h. We followed the ARRIVE guidelines (Kilkenny et al., 2010); hence, mice were randomized for behavioural tests, and the experimenter was blind to the genotype. All procedures were conducted in accordance with guidelines from the Canadian Council of Animal Care at the University of Western Ontario with an approved institutional animal protocol (2008-127).

4.4.2 Five-choice serial reaction time task training

A cohort of WT and CHRNA7−/− mice (n = 7 per genotype, 5–6 months old) was trained in the 5-CSRT task using the automated Bussey–Saksida Mouse Touch Screen System model 81426 (Campden Instruments, Lafayette, IN, USA). Schedules were designed, and data were collected using the abet ii touch software v.2.15 (Lafayette Instruments, Lafayette, IN, USA). Mice were trained to respond to the touch screen chambers using a previously described operant training procedure (Kolisnyk et al., 2013a, 2013b).

Training on the 5-CSRT task was performed as previously described (Romberg et al., 2011). Once the performance of a mouse reached criterion (80% accuracy, 20%

omissions for three consecutive days) at 4 s stimulus duration, stimulus duration was reduced to 2 s. After reaching criterion at a 2 s stimulus duration, the mouse was tested on probe trials.

4.4.3 Probe trial

To probe attention in mice, we used a previously described probe trial schedule with reduced stimulus durations (Romberg et al., 2011; Kolisnyk et al., 2013a). Mice were tested for 2 days at a given stimulus duration (1.5, 1, 0.8 or 0.6 s). Each day, sessions lasted 50 trials or 1 h. After each test, the animal was retested at the 2 s stimulus duration for 2 days, until the mice had been tested at all stimulus durations. The order of the probe trial sessions was semi-randomized using a Latin square method. Behavioural data were averaged over the 2 days of each stimulus duration.

4.4.4 Drug injections

For all drug experiments, mice were tested at the 0.6 s stimulus duration. The same mice used for the initial 5-CSRT experiments were used for the drug studies. Animals were injected 30 min before testing for the PHA-543,613 (Sigma-Aldrich, St Louis, MO, USA) and PNU-228,927 (Tocris Bioscience, Bristol, UK) experiments and 15 min before testing for the ABT-418 (Sigma-Aldrich) experiment (McGaughy et al., 1999b). Doses of PHA-543,613 [0.33, 1 and 3 mg·kg−1, i.p. (Acker et al., 2008)], PNU-282,927 [1, 3 and 5 mg·kg−1, i.p. (Hajos et al., 2005; Vicens et al., 2013)] and ABT-418 [0.04, 0.13 and 0.39 mg·kg−1, i.p. (McGaughy et al., 1999a)] were chosen based on previous studies. In control experiments, vehicle (saline) was injected. The order of drug injections was semi-randomized using a Latin square method. Between different doses in the drug injection experiments, mice were given two washout days during which they were baselined with a 2 s stimulus duration.

4.4.5 Analysis of 5-CSRT task

On all 5-CSRT task sessions, accuracy was calculated as the number of correct responses divided by the number of correct and incorrect responses (touches to a

wrong window while the correct stimulus was still displayed). Omissions were calculated as the total number of omitted trials divided by the number of total trials. Response latency was the time the mouse took to touch the correct stimulus after the onset of its display. Reward collection latency was defined as the time it took the mouse to enter the reward magazine following a correct response. A premature response was counted when the mouse touched one of the windows prior to the stimulus being displayed. Finally, a perseverative response was any identical response that occurred following a correct, incorrect or premature response.

4.4.6 Food intake in food-deprived mice

Feeding behaviour was analysed as previously described (Semenova and Markou, 2007). Naive groups of WT and CHRNA7−/− mice (n = 8 per genotype) were housed individually and were deprived of food overnight before the test. During the test, mice were placed in a clean cage and given 20 g of standard chow. Food intake was measured 20, 40, 60 and 80 min after the start of the test. Food intake was normalized to the body weight of the animals.

4.4.7 qPCR

Total RNA was extracted from freshly dissected PFC tissue, using the Aurum Total RNA for fatty and fibrous tissue kit (Bio-Rad Laboratories, Hercules, CA, USA); cDNA synthesis and quantitative PCR (qPCR) analysis of nicotinic receptor expression were performed as previously described (Guzman et al., 2011; Kolisnyk et al., 2013a).

4.4.8 Western Blotting

Western blotting was performed as previously described (Martins-Silva et al., 2011). For analysis of phospho-proteins in the PFC, mice were given i.p. drug injections and were killed 30 min later. Tissue was then homogenized in lysis buffer supplemented with protease and phosphatase inhibitor cocktails (Thermo Fisher Scientific, Waltham, MA, USA). The antibodies used were anti-vesicular ACh transporter (VAChT) (catalogue #139103; Synaptic Systems, Göttingen, Germany), anti-ChAT (catalogue #1DB-001-

0000849693; Millipore, Billerica, MA, USA), anti-synaptophysin (catalogue #S5768; Sigma-Aldrich), anti-ERK1/2 (catalogue #4695; Cell Signaling Technology, Danvers, MA, USA), anti-phospho-ERK1/2 (catalogue #4372; Cell Signaling Technology), anti-cFos (catalogue #4384; Cell Signaling Technology) and anti-β-actin (catalogue #ab49900; Abcam, Cambridge, UK). Band intensity was quantified using fluorochemq software (Thermo Fisher Scientific).

4.4.9 Statistical analyses

All data are expressed as mean ± SEM. sigmastat 3.5 (Systat Software, San Jose, CA, USA) was used for all statistical analysis. Comparisons between two experimental groups were made by Student's t-test. When several experimental groups or treatments were analysed, one-way ANOVA or two-way ANOVA with repeated-measures tests were used as required. Statistically significant effects were further analysed using Tukey's honestly significant difference post hoc tests. In all analyses, $P < 0.05$ was considered statistically significant.

4.5 Results

4.5.1 α7nAChR-null mice present normal acquisition on the 5-CSRT task

No difference between CHRNA7−/− mice and WT controls was observed in the number of sessions required to reach criterion at any of the pre-training phases for the 5-CSRT task [Fig. 4.1.1; RM-ANOVA: no effect of genotype, $F(1,14) = 2.814$, $P = 0.1156$; main effect of training phase, $F(4,14) = 104.3$, $P < 0.0001$; no interaction effect, $F(4,14) = 1.126$, $P = 0.3535$]. During training on the 5-CSRT task as well, CHRNA7−/− mice took as many sessions as WT controls to achieve criterion at both the 4 and 2 s stimulus durations [RM-ANOVA: no effect of genotype, $F(1,14) = 2.552$, $P = 0.1325$; main effect of stimulus duration, $F(1,14) = 57.78$, $P < 0.0001$; no interaction, $F(1,14) = 4.472$, $P = 0.0529$; Fig. 4.1.1B]. It should be noted that there was a strong tendency for the CHRNA7−/− mice to take longer to learn the task at the 4 s stimulus

duration, which may reflect previously documented impairments in procedural learning in these mice (Young et al., 2011).

4.5.2 α7nAChR-null mice have impaired sustained attention

Once mice reached criterion at 2 s stimulus duration, we assessed attention performance by using a probe trial, with reduced stimulus durations (1.5, 1, 0.8 and 0.6 s stimulus durations) as previously described (Romberg et al., 2011). Across all four stimulus durations, CHRNA7−/− mice performed similarly to controls in both total measures of omissions [RM-ANOVA: no effect of genotype, $F(1,36) = 3.235$, $P = 0.0972$; main effect of stimulus duration, $F(3,36) = 14.50$, $P < 0.001$; no interaction, $F(3,36) = 0.5136$, $P = 0.6755$; Figure 4.1.2A] and accuracy [RM-ANOVA: no effect of genotype, $F(1,36) = 0.06134$, $P = 0.8086$; main effect of stimulus duration, $F(3,36) = 9.208$, $P < 0.001$; no interaction, $F(3,36) = 0.6347$, $P = 0.5975$; Figure 4.1.2B]. To assess sustained attention, we analysed rate of omissions and response accuracy over blocks of 25 trials across the various stimulus durations of the probe trial experiment. As each probe trial session ends after 50 trials or 1 h, analysing blocks of 25 trials divided the performance between two halves: block A and block B. This procedure allowed us to determine if mice can sustain attention during the full period of the probe trial and maintain performance between the first and second periods of testing. Performance of control WT mice did not differ across the probe trial in terms of omissions [RM-ANOVA: no difference between blocks A and B, $F(1,6) = 1.904$, $P = 0.2168$; main effect of stimulus duration, $F(3,18) = 8.661$, $P < 0.001$; and no interaction, $F(3,18) = 0.4736$, $P = 0.7045$; Figure 4.1.2C) or accuracy [RM-ANOVA: no effect of blocks, $F(1,6) = 4.319$, $P = 0.0829$; main effect of stimulus duration, $F(3,18) = 4.897$, $P = 0.0166$; and no interaction, $F(3,18) = 0.4947$, $P = 0.6905$; Figure 4.1.2D]. In contrast, CHRNA7−/− mice displayed increased omission errors in the second half of the probe trial experiment compared with the first half [RM-ANOVA: main effect of block, $F(1,6) = 20.59$, $P < 0.001$; main effect of stimulus duration, $F(3,18) = 9.471$, $P < 0.001$; and main interaction effect, $F(3,18) = 12.13$, $P < 0.001$; Figure 4.1.2E). Post hoc analysis confirmed that CHRNA7−/− mice had significantly more omission errors during the second half of the task at both the 0.8 and 0.6 s

stimulus durations, suggesting that these mice display impaired ability to sustain attention. Interestingly, CHRNA7−/− mice did not present accuracy impairments across the two blocks [RM-ANOVA: no effect of block, $F(1,6) = 1.348$, $P = 0.2897$; main effect of stimulus duration, $F(3,18) = 5.877$, $P = 0.0056$; and no interaction effect, $F(3,18) = 0.6404$, $P = 0.5989$; Figure 4.1.2F].

Increases in omission on the 5-CSRT task have been proposed to be due to lack of attention, lack of motivation or motor impairments (Robbins, 2002). Given that CHRNA7−/− mice were no different from controls in terms of latency to respond to stimulus or latency to collect the reward, it is unlikely that motivation or motor impairments are causing the deficits in sustained attention (Robbins, 2002; Spinelli et al., 2004). To address this, we measured several other parameters to test for motivational or aberrant behaviour in CHRNA7−/− mice. α7nAChR-null mice showed no difference in latency to respond to the stimulus [RM-ANOVA: no effect of genotype, $F(1,36) = 0.01533$, $P = 0.9035$; no effect of stimulus duration, $F(3,36) = 2.003$, $P = 0.1310$; no interaction, $F(3,36) = 1.223$, $P = 0.3154$; Figure 4.1.3A]. We then assessed response latency across blocks of trials to determine if the sustained attention deficits in CHRNA7−/− mice may be due to delayed responsiveness. Response latency did not differ across blocks for either the WT [RM-ANOVA: no effect of block, $F(1,6) = 0.07218$, $P = 0.7972$; no effect of stimulus duration, $F(3,18) = 2.749$, $P = 0.0792$; and no interaction effect, $F(3,18) = 0.7886$, $P = 0.5159$; Figure 4.1.3B] or CHRNA7−/− mice [RM-ANOVA: no effect of block, $F(1,6) = 0.2481$, $P = 0.6362$; no effect of stimulus duration, $F(3,18) = 2.2557$, $P = 0.1166$; and no interaction effect, $F(3,18) = 1.490$, $P = 0.2501$; Figure 4.1.3C]. We also assessed the time to retrieve their reward following a correct response [RM-ANOVA, no effect of genotype, $F(1,36) = 0.2025$, $P = 0.6607$; no effect of stimulus duration, $F(3,36) = 1.153$, $P = 0.3410$; no interaction, $F(3,36) = 0.2954$, $P = 0.8284$; Figure 4.1.3D] when compared with WT controls. Furthermore, we assessed reward latency across blocks of trials, and neither the WT [RM-ANOVA: no effect of block, $F(1,6) = 2.345$, $P = 0.1766$; no effect of stimulus duration, $F(3,18) = 2.176$, $P = 0.1262$; and no interaction effect, $F(3,18) = 0.5404$, $P = 0.6677$; Figure 4.1.3E) nor CHRNA7−/− mice [RM-ANOVA, no effect of block, $F(1,6) = 0.02162$, $P = 0.8879$; no effect of stimulus duration, $F(3,18) = 0.6190$, $P = 0.6617$; and no

interaction effect, $F(3,18) = 0.3581$, $P = 0.7839$; Figure 4.1.3F) showed alteration in reward collection latency. To test whether CHRNA7−/− mice differ from WT controls in satiety, we measured food intake following food restriction in a group of naive mice. Compared with controls, CHRNA7−/− mice did not differ in food consumption over the course of the test [RM-ANOVA: no effect of genotype, $F(1,48) = 1.280$, $P = 0.2800$; main effect of time, $F(4,48) = 73.88$, $P < 0.001$; and no interaction effect, $F(4,48) = 1.296$, $P = 0.2849$; Figure 4.1.3G]. This is in line with previous work showing that these mice have normal motivation (Hoyle et al., 2011) and suggests that CHRNA7−/− mice have specific deficits in sustained attention.

Impulsivity and compulsivity were also assessed in CHRNA7−/− mice during the probe trial experiment. Compared with controls, CHRNA7−/− mice were no different in terms of premature responses, a measure of impulsivity [RM-ANOVA: no effect of genotype, $F(1,36) = 0.9222$, $P = 0.3575$; no effect of stimulus duration, $F(3,36) = 0.4541$, $P = 0.7161$; no interaction effect, $F(3,36) = 0.09521$, $P = 0.9621$; Fig. 4.1.4A, B], or perseverative responses, a measure of compulsive behaviour [RM-ANOVA: no effect of genotype, $F(1,36) = 0.04477$, $P = 0.8363$, main effect of stimulus duration, $F(3,36) = 4.105$, $P = 0.0140$; no interaction effect, $F(3,36) = 0.8660$, $P = 0.4685$, Fig. 4.1.4C, D].

The ability to release normal levels of ACh is critical to attention (Kolisnyk et al., 2013a,b); therefore, we investigated expression levels of the cholinergic machinery in the PFC of CHRNA7−/− mice. Compared with WT controls, CHRNA7−/− mice showed no significant change in expression of the VAChT [$t(4) = 0.375$, $P = 0.7291$] or ChAT. The sustained attention deficits in CHRNA7−/− mice are therefore not a result of an inherent dysfunction in the machinery required for ACh release (Figure 4.1.5A).

α7 nicotinic ACh receptor deletion has been suggested to cause compensatory changes in other nicotinic receptors during development (Yu et al., 2007). To determine if the PFC of adult CHRNA7−/− mice displayed altered expression of nicotinic receptors, we examined their expression by qPCR analysis. The PFC of CHRNA7−/− mice showed no significant change in the expression of CHRNA4 [$t(8) = 1.104$, $P = 0.3016$] or of CHRNB2 expression [$t(8) = 0.4893$, $P = 0.6378$]. In addition, we evaluated the

expression of the enzyme AChE and observed no significant difference between genotypes [$t(8) = 0.0409$, $P = 0.9684$]. As expected, we did not detect CHRNA7 expression in CHRNA7−/− animals (Figure 4.1.5B).

To evaluate the biochemical correlates of neuronal activity in the PFC of CHRNA7−/− mice, we determined protein levels of the immediate-early gene cFos, a known marker of activated neurons. Compared with the WT control, CHRNA7−/− mice showed no significant change in cFos protein levels [$t(4) = 0.779$, $P = 0.4792$, Figure 4.1.5C]. Moreover, to test if the CHRNA7−/− had impaired activation of relevant second messenger signalling cascades involved with nicotinic response in attention (Wallace and Porter, 2011b), we evaluated the phosphorylation status of ERK1/2 and observed no significant difference between genotypes [$t(4) = 0.331$, $P = 0.7575$, Figure 4.1.5D].

Figure 4.1.1. Pre-training on the 5-CSRT task. (a) Sessions to criteria during pretraining for the 5-CSRT task. Numbers designate phases of the pre-training (1 – ‘habituation’, 2 – ‘initial touch’, 3 – ‘must touch’, 4 – ‘must initiate’, 5 – ‘punish incorrect’). (b) Sessions to criteria during training on the 5-CSRT task. (Data are presented as mean ± SEM.).

Figure 4.1.1. Pre-training on the 5-CSRT task.

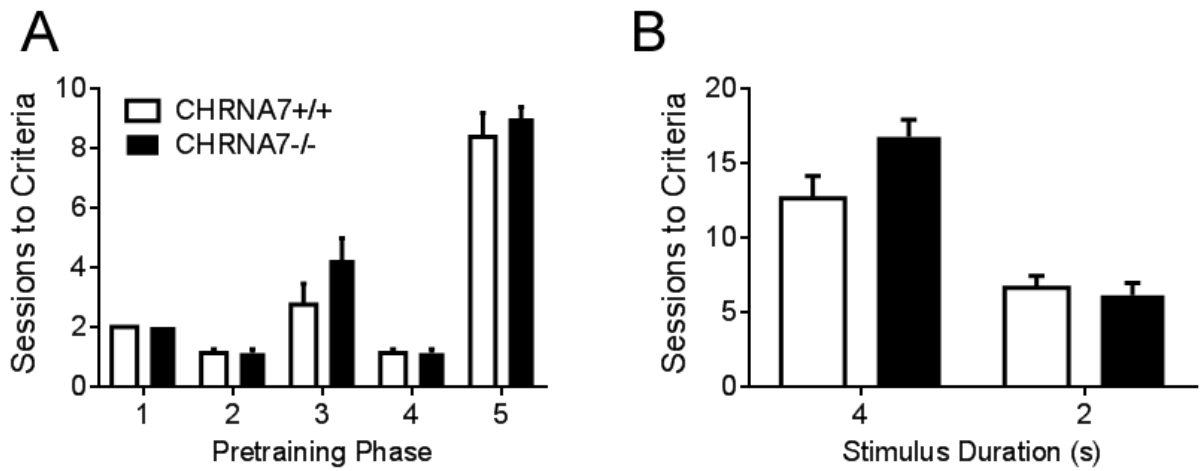


Figure 4.1.2. CHRNA7−/− mice have impaired sustained attention. Comparison between genotypes of (A) omissions and (B) accuracy during the probe trial experiment using the 5-CSRT task. (C) Omissions and (D) accuracy across bins of 25 trials in WT mice. (E) Omissions and (F) accuracy across bins of 25 trials in CHRNA7−/− mice (data are presented as mean ± SEM; *P < 0.05,**P < 0.01, ***P < 0.001).

Figure 4.1.2. CHRNA7−/− mice have impaired sustained attention.

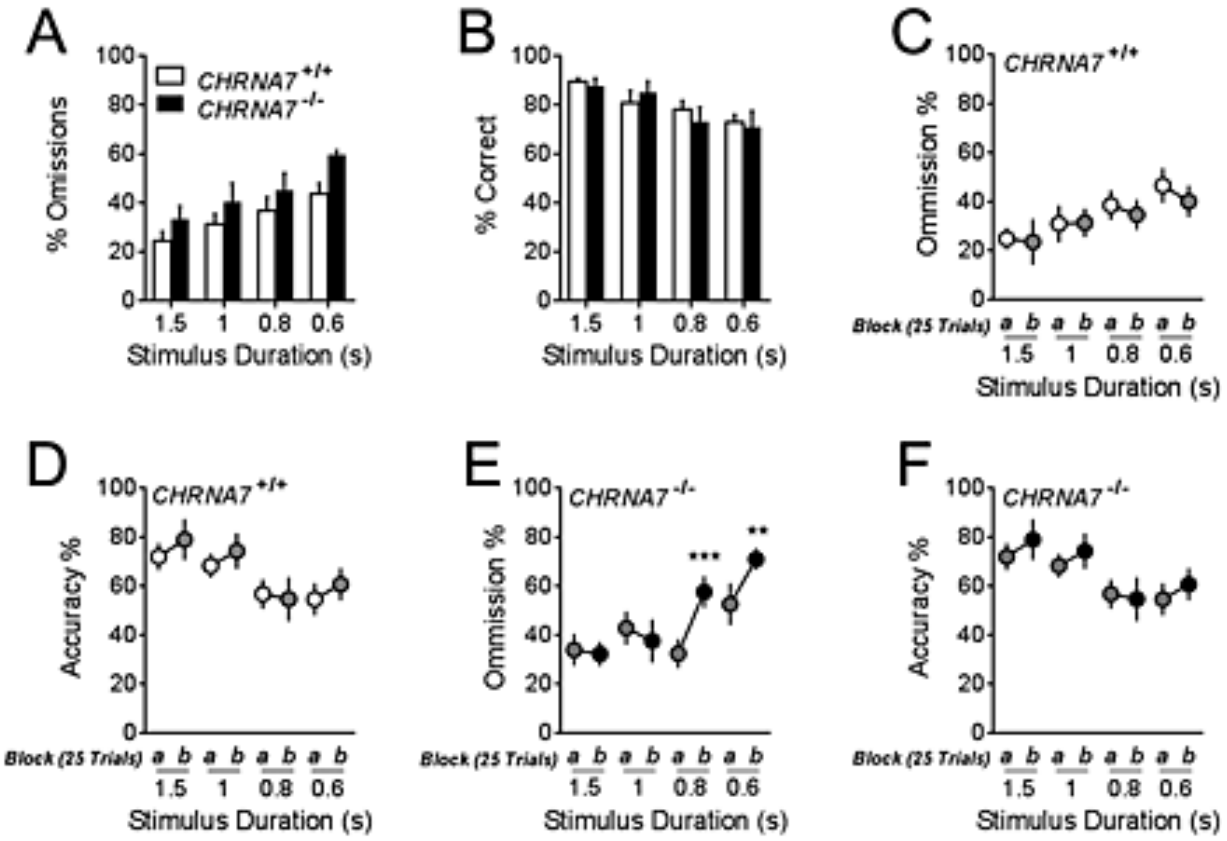


Figure 4.1.3. CHRNA7−/− mice have normal motivation and motor function during the 5-CSRT task. Comparison between genotypes of response latencies (A). Response latencies across bins of 25 trials in wild-type (B) and CHRNA7−/− mice (C). Comparison between genotypes of reward collection latencies (D). Reward collection latencies across bins of 25 trials in wild-type (E) and CHRNA7−/− mice (F). (G) Food consumption following food deprivation as a measure of motivation (data are presented as mean ± SEM).

Figure 4.1.3. CHRNA7−/− mice have normal motivation and motor function during the 5-CSRT task.

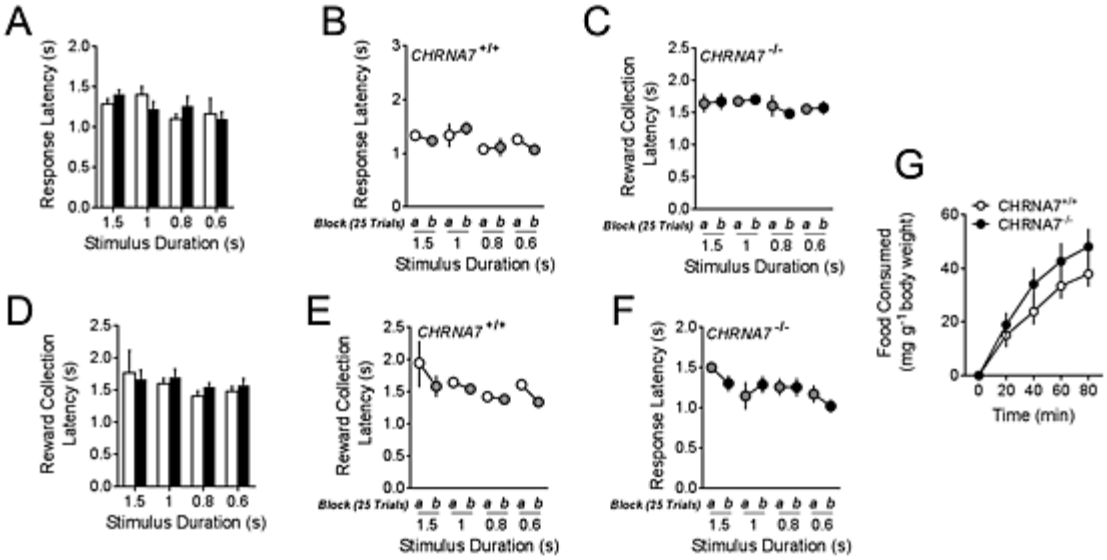


Figure 4.1.4. Response patterns did not differ in α7nAChR null mice on the 5-CSRT task probe trial. Premature (a) and perseverative (b) responses between WT (clear bars) and CHRNA7−/− mice (dark bars). (Data are presented as mean ± SEM.)

Figure 4.1.4. Response patterns did not differ in α7nAChR null mice on the 5-CSRT task probe trial.

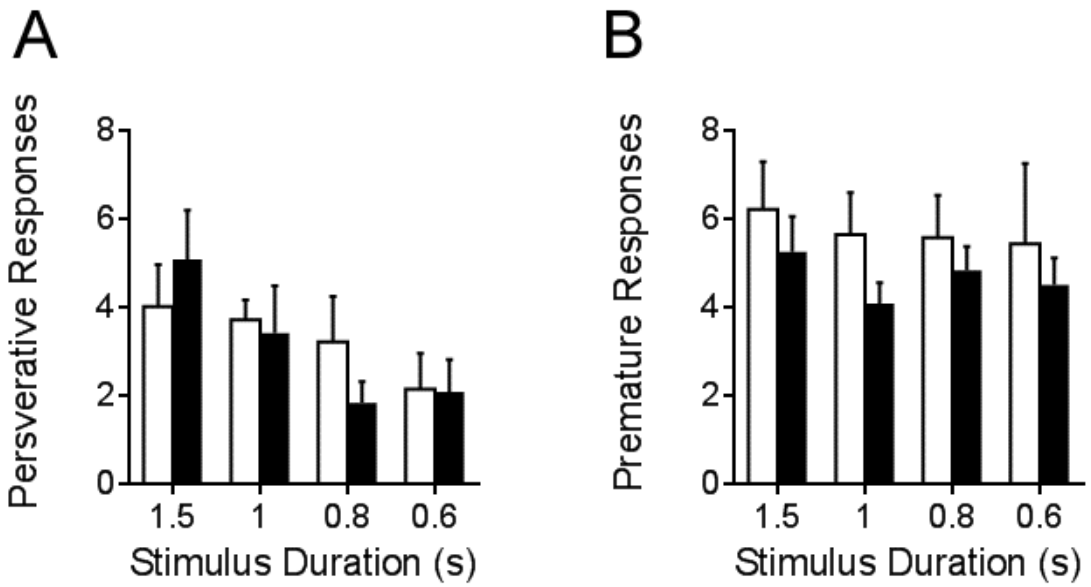
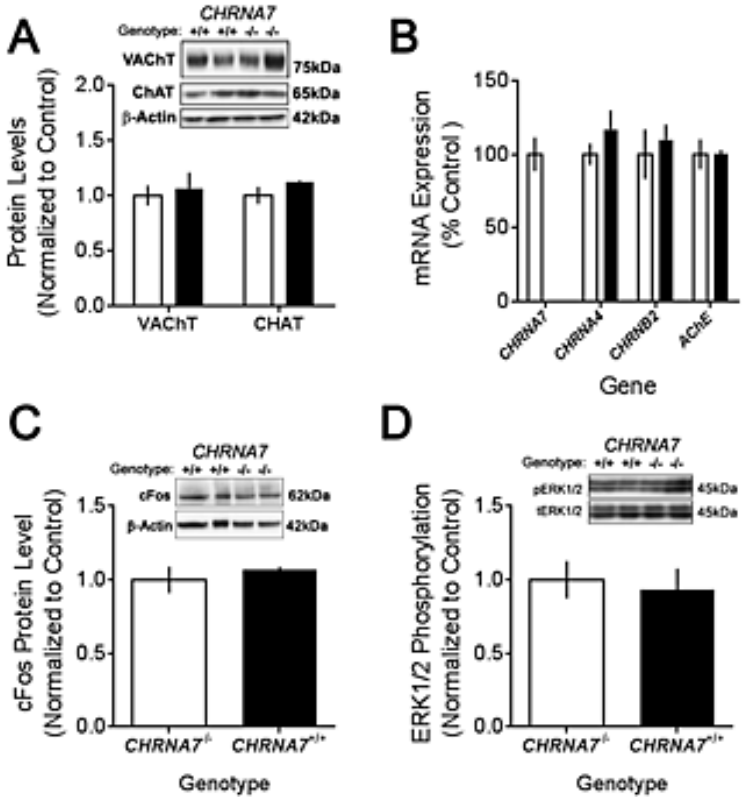


Figure 4.1.5. Evaluation of the expression of cholinergic markers and relevant signalling pathways in the PFC of CHRNA7−/− mice. (A) Immunoblot of VAChT and ChAT expression in the PFC. (B) qPCR expression of nicotinic receptors and AChE in the PFC. (C) cFos protein levels and (D) ERK1/2 phosphorylation in the PFC (data are presented as mean ± SEM).

Figure 4.1.5. Evaluation of the expression of cholinergic markers and relevant signalling pathways in the PFC of CHRNA7−/− mice.



4.5.3 Effect of α7nAChR agonists on attention

To evaluate acute roles of α7nAChR in regulating sustained attention behaviour, we investigated two selective α7nAChR agonists, PHA-543,613 (Acker et al., 2008) and PNU-282,987 (Hajos et al., 2005), in WT mice using the 5-CSRT task. Mice were tested at a 0.6 s stimulus duration, which represents a cognitively demanding version of the task (Romberg et al., 2011; Kolisnyk et al., 2013a). PHA-543,613 significantly improved rate of omissions [RM-ANOVA: main effect of dose, $F(3,18) = 12.52$, $P < 0.001$; Figure 4.2.1A], with post hoc analysis confirming that the 1 mg·kg−1 dose significantly improved performance over saline. Conversely, PHA-543,613 significantly altered response accuracy in higher doses [RM-ANOVA: main effect of dose, $F(3,18) = 12.55$, $P < 0.001$; Figure 4.2.1B]. Post hoc analysis revealed that at the highest dose tested (3 mg·kg−1), PHA-543,613-injected mice performed significantly worse than mice injected with saline. PHA-543,613 did not significantly alter response latency [RM-ANOVA: no effect of dose, $F(3,18) = 1.568$, $P = 0.2318$, Fig. 4.2.2A] or reward collection latency [RM-ANOVA: no effect of dose, $F(3,18) = 0.7517$, $P = 0.5382$; Fig. 4.2.2B]. In addition, PHA-543,613 did not alter premature [RM-ANOVA: no effect of dose, $F(3,18) = 0.7599$, $P = 0.4930$) nor perseverative responses [RM-ANOVA: no effect of dose, $F(3,18) = 0.1404$, $P = 0.8821$, Fig. 4.2.2C, D]. To address the effects of PHA-543,613 on sustained attention, we analysed performance of mice over blocks of 25 trials and observed that PHA-543,613 did not significantly alter sustained omissions between the two blocks of testing [RM-ANOVA: main effect of dose, $F(3,36) = 13.20$, $P < 0.0001$; no effect of block, $F(1,12) = 0.7069$, $P = 0.4327$; and no interaction, $F(3,36) = 1.288$, $P = 0.3088$; Figure 4.2.1C], nor did it alter accuracy in WT mice [RM-ANOVA: main effect of dose, $F(3,36) = 14.63$, $P < 0.0001$; no effect of block, $F(1,12) = 1.729$, $P = 0.101$; and no interaction effect, $F(3,18) = 0.0713$, $P = 0.9749$; Figure 4.2.1D). At 1 mg·kg−1, the percentage of omissions seemed to be slightly reduced in the second block, suggesting modest improvement in the performance.

The second α7nAChR agonist tested, PNU-282,987, also significantly improved rate of omissions [RM-ANOVA: main effect of dose, $F(3,18) = 2.767$, $P = 0.0437$, Figure 4.2.1E], with post hoc analysis confirming that both the 1 and 3 mg·kg−1 doses

significantly improved performance over saline. Conversely, PNU-282,978 significantly altered response accuracy [RM-ANOVA: main effect of dose, $F(3,18) = 5.637$, $P = 0.0066$; Figure 4.2.1F). Post hoc analysis revealed that at the highest dose tested (5 mg·kg−1) mice injected with PNU-282,978 performed significantly worse than mice injected with saline. PNU-282,978 did not significantly alter response latency [RM-ANOVA: no effect of dose, $F(3,18) = 0.9985$, $P = 0.4018$; Fig. 4.2.2E] nor reward collection latency [RM-ANOVA: no effect of dose, $F(3,18) = 1.131$, $P = 0.3375$; Figure 4.3.2.2.1F]. PNU-282,978 did not alter the number of premature [RM-ANOVA: no effect of dose, $F(3,18) = 3.015$, $P = 0.1157$] or perseverative responses [RM-ANOVA: no effect of dose, $F(3, 18) = 0.4522$, $P = 0.6707$, Fig. 4.2.2G, H]. In terms of the effects of PNU-282,978 on sustained attention, analysis of injected mice over two blocks of 25 trials showed that PNU-282,978 did alter the rate of omissions, with 3 mg·kg−1 improving omission rates over the two blocks [RM-ANOVA: main effect of dose, $F(3,36) = 6.095$, $P = 0.0031$; no effect of block, $F(1,12) = 0.5240$, $P = 0.4761$; and main interaction effect, $F(3,36) = 3.218$, $P = 0.0407$; Figure 4.2.1G], but did not alter sustained accuracy [RM-ANOVA: main effect of dose, $F(3,36) = 6.044$, $P = 0.0019$; no effect of block, $F(1,12) = 0.222$, $P = 0.6458$; and no interaction effect, $F(3,18) = 0.0701$, $P = 0.9755$; Figure 4.2.1H].

To evaluate the biochemical correlates of acute α7nAChR agonist activation in the PFC, we injected PHA-543,613 on WT mice and determined protein levels of cFos and the phosphorylation status of ERK1/2. These experiments used a separate cohort of mice, which were injected with drug or saline and then killed 30 min later and had their PFC dissected to obtain protein extracts. Compared with saline, PHA-543,613 injected in mice led to a significant increase in the levels of cFos protein in their PFC [one-way ANOVA: main effect of dose, $F(2,6) = 7.404$, $P = 0.0240$; Figure 4.2.1I], with post hoc analysis showing that cFos levels were increased at both doses of PHA-543,613. Similarly, injections of PHA-543,613 significantly increased ERK1/2 phosphorylation levels in a dose-dependent way [one-way ANOVA: main effect of dose, $F(2,6) = 28.80$, $P < 0.001$; Figure 4.2.1J].

Figure 4.2.1 α7nAChR agonists improve attention in wild-type mice. (A) Omission and (B) accuracy following injections of PHA-543,613 in WT mice. (C) Omissions and (D) accuracy over bins of 25 trials following administration of PHA-543,613. (E) Omission and (F) accuracy following injections of PNU-282,987 in WT mice. (G) Omissions and (H) accuracy over bins of 25 trials following administration of PNU-282,987. (I) cFos protein levels and (J) ERK1/2 phosphorylation following injection of PHA-543,613 (data are presented as mean ± SEM; *P < 0.05,**P < 0.01, ***P < 0.001).

Figure 4.2.1 α7nAChR agonists improve attention in wild-type mice.

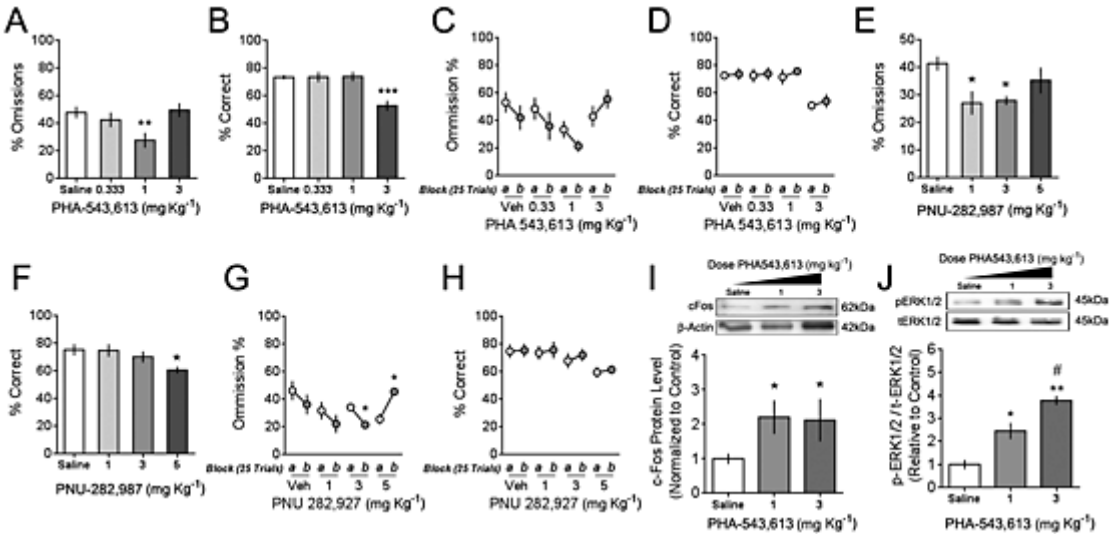
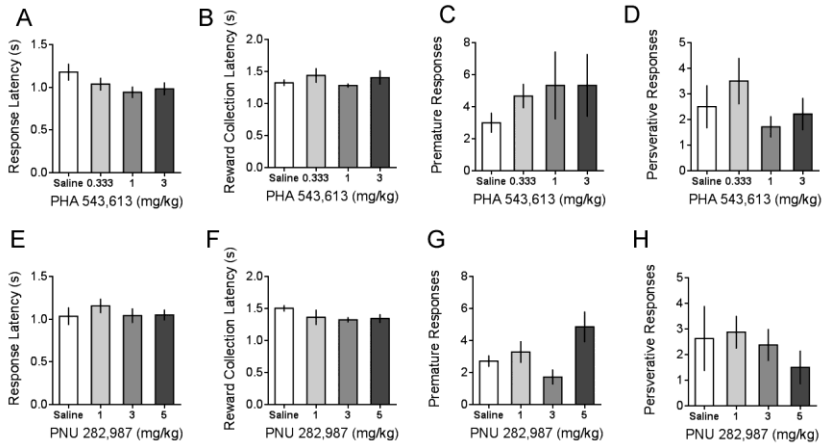


Figure 4.2.2 α7nAChR agonists did not alter response patterns in wild-type mice. Premature responses (a), perseverative responses (b), response (c) and reward collection (d) latencies following PHA-543,613 injections in WT mice. Premature responses (e), perseverative responses (f), response (g) and reward collection (h) latencies following PNU-282,927 injections in WT mice. (Data are presented as mean ± SEM.)

Figure 4.2.2 α7nAChR agonists did not alter response patterns in wild-type mice.



4.5.4 Positive and negative effects of α7nAChR agonists are abolished in CHRNA7−/− mice

To confirm the specificity of both PHA-543,613 and PNU-282,987 for α7nAChRs, we administered both compounds to CHRNA7−/− mice prior to testing them on the 5-CSRT task with a 0.6 s stimulus duration. Compared with saline, PHA-543,613 had no effect on the performance of the mice at any dose tested. PHA-543,613 did not alter rate of omissions [RM-ANOVA: no effect of dose, $F(3,18) = 1.528$, $P = 0.2515$; Figure 4.3.1A] or response accuracy [RM-ANOVA: no effect of dose, $F(3,18) = 0.1121$, $P = 0.8733$; Figure 4.3.1B], response latency [RM-ANOVA: no effect of dose, $F(3,18) = 0.2490$, $P = 0.7348$; Figure 4.3.2A] or reward collection latency [RM-ANOVA: no effect of dose, $F(3,18) = 0.3018$, $P = 0.6621$; Figure 4.3.2B]. PHA-543,613 did not alter the number of premature [RM-ANOVA: no effect of dose, $F(3,18) = 1.104$, $P = 0.3579$] or perseverative responses [RM-ANOVA: no effect of dose, $F(3,18) = 2.101$, $P = 0.1738$, Figure 4.3.2C, D]. Furthermore, when we analysed performance over blocks of 25 trials, we observed that PHA-543,613 did not alter impaired omission deficit of CHRNA7−/− mice at 0.6 s, which remained significantly higher in block B across all doses [RM-ANOVA: no effect of dose, $F(3,36) = 1.528$, $P = 0.2414$; main effect of block, $F(1,12) = 14.89$, $P = 0.0084$; and no interaction $F(3,12) = 0.2209$, $P = 0.8806$; Figure 4.3.1C]. PHA-543,613 did not alter sustained accuracy in CHRNA7−/− mice either [RM-ANOVA: no effect of dose, $F(3,36) = 0.2017$, $P = 0.8945$; no effect of block, $F(1,12) = 0.00701$, $P = 0.9343$; and no interaction, $F(3,36) = 0.02177$, $P = 0.9956$; Figure 4.3.1D].

As with the PHA-543,613, PNU-282,987 had no effect on the performance of CHRNA7−/− mice at any of the tested doses. The drug did not alter rate of omissions [RM-ANOVA: no effect of dose, $F(3,18) = 0.1515$, $P = 0.9277$; Figure 4.3.1E], response accuracy [RM-ANOVA: no effect of dose, $F(3,18) = 0.1458$, $P = 0.9310$; Figure 4.3.1F], response latency [RM-ANOVA: no effect of dose, $F(3,18) = 0.0586$, $P = 2.808$, Figure 4.3.2E] or reward collection latency [RM-ANOVA: no effect of dose, $F(3,18) = 0.8089$, $P = 0.4603$, Figure 4.3.2F]. PNU-282,978 did not alter the number of premature [RM-ANOVA: no effect of dose, $F(3,18) = 0.2767$, $P = 0.8316$] or perseverative responses [RM-ANOVA: no effect of dose, $F(3,18) = 0.2218$, $P = 0.8036$, Figure 4.3.2G, H]. Also,

analysis of performance of injected mice over blocks of 25 trials showed that the α7nAChR agonist had no effect on the impaired omission deficit that we consistently observed on CHRNA7−/− mice [RM-ANOVA: main effect of block, $F(1,6) = 9.112$, $P = 0.0234$; no effect of dose, $F(3,18) = 0.2542$, $P = 0.8573$; and no interaction, $F(3,18) = 0.2546$, $P = 0.8570$; Figure 4.3.1G] nor on sustained accuracy [RM-ANOVA: no effect of blocks, $F(1,6) = 0.0258$, $P = 0.9945$; no effect of dose, $F(3,18) = 1.123$, $P = 0.3729$; and no interaction, $F(3,18) = 0.2665$, $P = 0.8491$; Figure 4.3.1H]. Taken together, these results demonstrate that modulation of attention performance on the 5-CSRT task by both PHA-543,613 and PNU-282,987 depends on their activity on α7nAChR.

Additionally, to confirm the selectivity of the molecular changes observed following PHA-543,613 administration in WT mice, we injected CHRNA7−/− mice with the highest dose of the drug (3 mg·kg−1) and then, 30 min later, measured the effects on cFos protein levels and ERK1/2 phosphorylation in the PFC. Unlike PHA-543,613-injected WT mice, CHRNA7−/− mice exhibited no change in cFos levels [$t(4) = 0.387$, $P = 0.7186$; Figure 4.3.1I] or ERK1/2 phosphorylation [$t(4) = 0.1029$, $P = 0.9230$; Figure 4.3.1J], suggesting that both the behaviour and molecular effects of the drug are specific to activation of α7nAChR.

Figure 4.3.1. α7nAChR agonists do not alter attention in mice lacking α7nAChR. (A) Omission and (B) accuracy following injections of PHA-543,613 in CHRNA7−/− mice. (C) Omissions and (D) accuracy over bins of 25 trials following administration of PHA-543,613 in CHRNA7−/− mice. (E) Omission and (F) accuracy following injections of PNU-282,987 in CHRNA7−/− mice. (G) Omissions and (H) accuracy over bins of 25 trials following administration of PNU-282,987. (I) cFos protein levels and (J) ERK1/2 phosphorylation following injection of PHA-543,613 (data are presented as mean ± SEM; *P < 0.05,**P < 0.01, ***P < 0.001).

Figure 4.3.1. α7nAChR agonists do not alter attention in mice lacking α7nAChR.

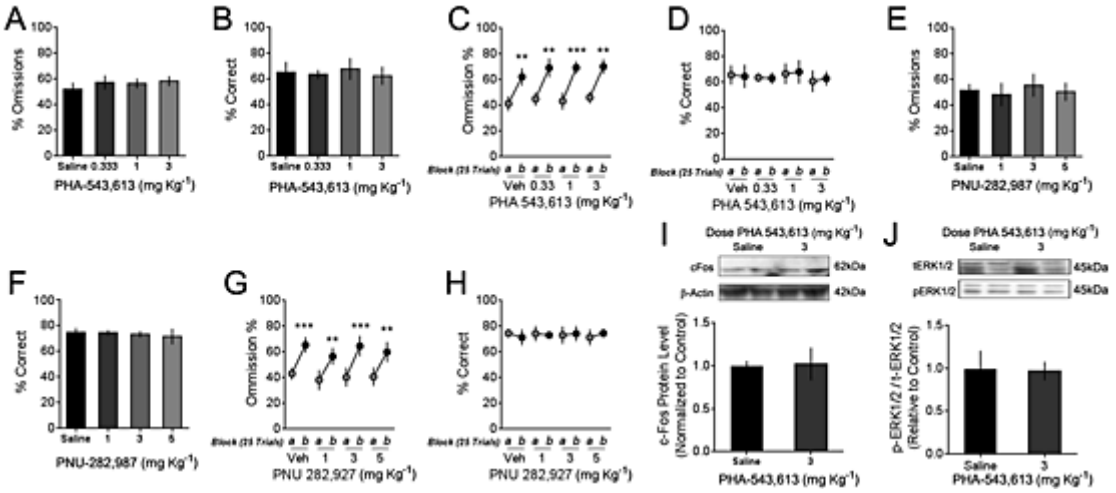
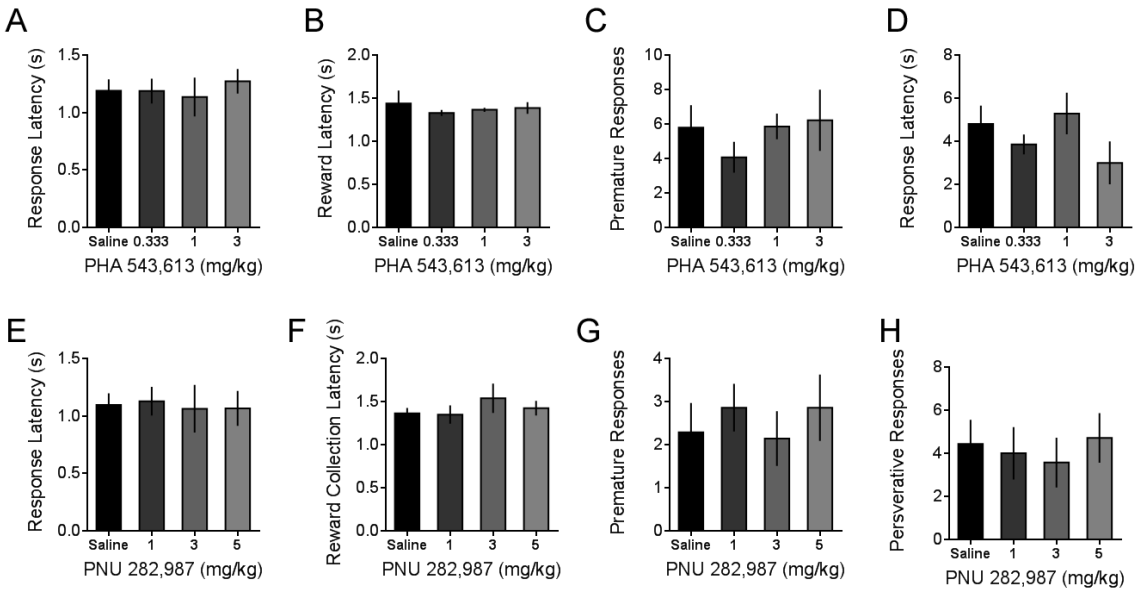


Figure 4.3.2. α7nAChR agonists did not alter response patterns in CHRNA7-null mice. Premature responses (a), perseverative responses (b), response (c) and reward collection (d) latencies following PHA-543,613 injections in CHRNA7−/− mice. Premature responses (e), perseverative responses (f), response (g) and reward collection (h) latencies following PNU-282,927 injections in CHRNA7−/− mice. (Data are presented as mean ± SEM.).

Figure 4.3.2. α7nAChR agonists did not alter response patterns in CHRNA7-null mice.



4.5.5 The β2nAChR agonist ABT-418 improves attention

In order to explore the relationship between distinct types of nicotinic receptors on attentional performance, we used ABT-418, a β2nAChR agonist, and treated WT mice that were tested with the 0.6 s stimulus duration paradigm. Injections of ABT-418 were able to significantly improve both rate of omissions [RM-ANOVA: main effect of dose, $F(3,18) = 4.544$, $P = 0.0132$; Figure 4.4.1A] and response accuracy [RM-ANOVA: main effect of dose, $F(3,18) = 6.950$, $P = 0.0020$; Figure 4.4.1B] without altering response latency [RM-ANOVA: no effect of dose, $F(3,18) = 0.06377$, $P = 0.9014$; Figure 4.4.2A] or reward collection latency [RM-ANOVA: no effect of dose, $F(3,18) = 0.2936$, $P = 0.8797$; Figure 4.4.2B]. ABT-418 did not alter the number of premature [RM-ANOVA: no effect of dose, $F(3,18) = 1.228$, $P = 0.3103$] or perseverative responses [RM-ANOVA: no effect of dose, $F(3,18) = 0.3062$, $P = 0.6764$, Figure 4.4.2C, D]. To evaluate the effects of ABT-418 on sustained attention, we analysed accuracy and omissions across blocks of 25 trials. ABT-418 did not significantly alter sustained omissions across blocks for WT mice [RM-ANOVA: no effect of block, $F(1,6) = 0.6582$, $P = 0.6013$; main effect of dose, $F(3,18) = 0.2542$, $P = 0.8573$; and no interaction, $F(3,18) = 0.6582$, $P = 0.5847$; Figure 4.4.1C]. ABT-418 had no effect on sustained accuracy in WT mice, with the improvements brought on by the drug spanning across both blocks of trials [RM-ANOVA: no effect of block, $F(1,6) = 0.7267$, $P = 0.4083$; main effect of dose, $F(3,18) = 7.744$, $P = 0.0003$; and no interaction $F(3,18) = 1.084$, $P = 0.3662$; Figure 4.4.1D).

Given that nicotinic receptors may be expressed in similar populations of neurons and could crosstalk (Azam et al., 2003), we evaluated whether β2nAChR receptor activation could impact attention in mice lacking α7nAChR. As with the WT mice, ABT-418 was able to significantly improve both omissions [RM-ANOVA: main effect of dose, $F(3,18) = 5.466$, $P = 0.0066$; Figure 4.4.3A] and accuracy [RM-ANOVA: main effect of dose, $F(3,18) = 3.383$, $P = 0.0373$; Figure 4.4.3B] in CHRNA7−/− mice, without altering response latency [RM-ANOVA: no effect of dose, $F(3,18) = 1.622$, $P = 0.2495$; Figure 4.4.4A] or reward collection latency [RM-ANOVA: no effect of dose, $F(3,18) = 0.8793$, $P = 0.4359$; Figure 4.4.4B]. ABT-418 did not alter the number of premature [RM-

ANOVA: no effect of dose, $F(3,18) = 0.1450$, $P = 0.81133$] or perseverative responses [RM-ANOVA: no effect of dose, $F(3,18) = 0.1254$, $P = 0.8336$; Figure 4.4.4C, D]. Importantly, ABT-418 was able to reverse the sustained attention deficits observed in CHRNA7−/− mice (Figure 4.4.3C) and improved the sustained omission deficits in these mice [RM-ANOVA: main effect of block, $F(1,6) = 11.82$, $P = 0.0138$; main effect of dose, $F(3,18) = 7.640$, $P = 0.0017$; and no interaction effect, $F(3,18) = 1.707$, $P = 0.2013$; Figure 4.4.3C]. Post hoc analysis revealed that this occurred even at the lowest dose administered. Sustained accuracy was not altered [RM-ANOVA: no effect of block, $F(1,6) = 0.1284$, $P = 0.7324$; main effect of dose, $F(3,18) = 5.017$, $P = 0.0106$; and no interaction, $F(3,18) = 1.054$, $P = 0.3929$; Figure 4.4.3D].

To determine the biochemical correlates of treatment with ABT-418 on CHRNA7−/− mice, we injected a new cohort of CHRNA7−/− mice with 0.39 mg·kg−1 of ABT-418 and 30 min later evaluated cFos and ERK1/2 phosphorylation levels in the PFC of the mice. Compared with saline-injected mice, CHRNA7−/− mice injected with ABT-418 showed a significant increase in cFos protein levels 30 min after injection [$t(4) = 5.610$, $P = 0.0050$; Figure 4.4.3E]. ABT-418 was also able to significantly increase ERK1/2 phosphorylation levels in the PFC of mice lacking α7nAChR [$t(4) = 5.300$, $P = 0.0061$; Figure 4.4.3F].

Importantly, given that the mice had been exposed to the task numerous times, we evaluated the performance of the mice over the course of the various injections in order to ensure that the improvements brought on by the ABT-418 were not due to the mice becoming better at the task. We compared the performance (both rates of omission and accuracy) of the mice from the vehicle injections of each drug experiment with their naive performance (the performance at a 0.6 s stimulus duration during the probe trial experiments). Both the WT {omission [one-way ANOVA: no effect of treatment, $F(6,18) = 0.7467$, $P = 0.4692$; Figure 4.4.57A] and accuracy [one-way ANOVA: no effect of treatment, $F(6,18) = 0.6749$, $P = 0.6716$; Figure 4.4.57B]} and CHRNA7−/− mice {omission [one-way ANOVA: no effect of treatment, $F(6,18) = 2.565$, $P = 0.1154$; Figure 4.4.57C] and accuracy [one-way ANOVA: no effect of treatment, $F(6,18) = 1.005$, $P = 0.3876$; Figure 4.4.57D]} demonstrated no significant change in performance in both omissions and accuracy from their naive performance across all injections.

Figure 4.4.1. β2nAChR agonists improve attention in wild-type mice. (A) Omission and (B) accuracy following injections of ABT-418 in WT mice. (C) Omissions and (D) accuracy over bins of 25 trials following administration of ABT-418 in WT mice (data are presented as mean ± SEM; *P < 0.05,**P < 0.01.

Figure 4.4.1. β2nAChR agonists improve attention in wild-type mice.

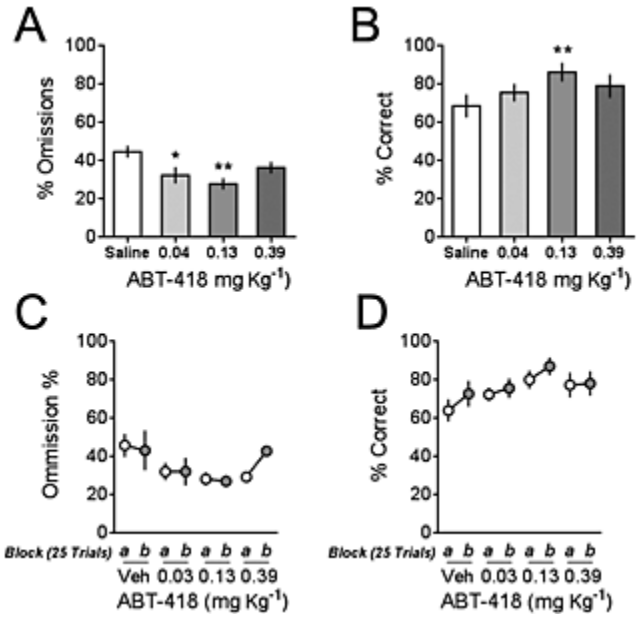


Figure 4.4.2. ABT-418 did not alter response patterns in wild-type mice. Premature responses (a), perseverative responses (b), response (c) and reward collection (d) latencies following ABT-418 injections in WT mice. (Data are presented as mean ± SEM.).

Figure 4.4.2. ABT-418 did not alter response patterns in wild-type mice.

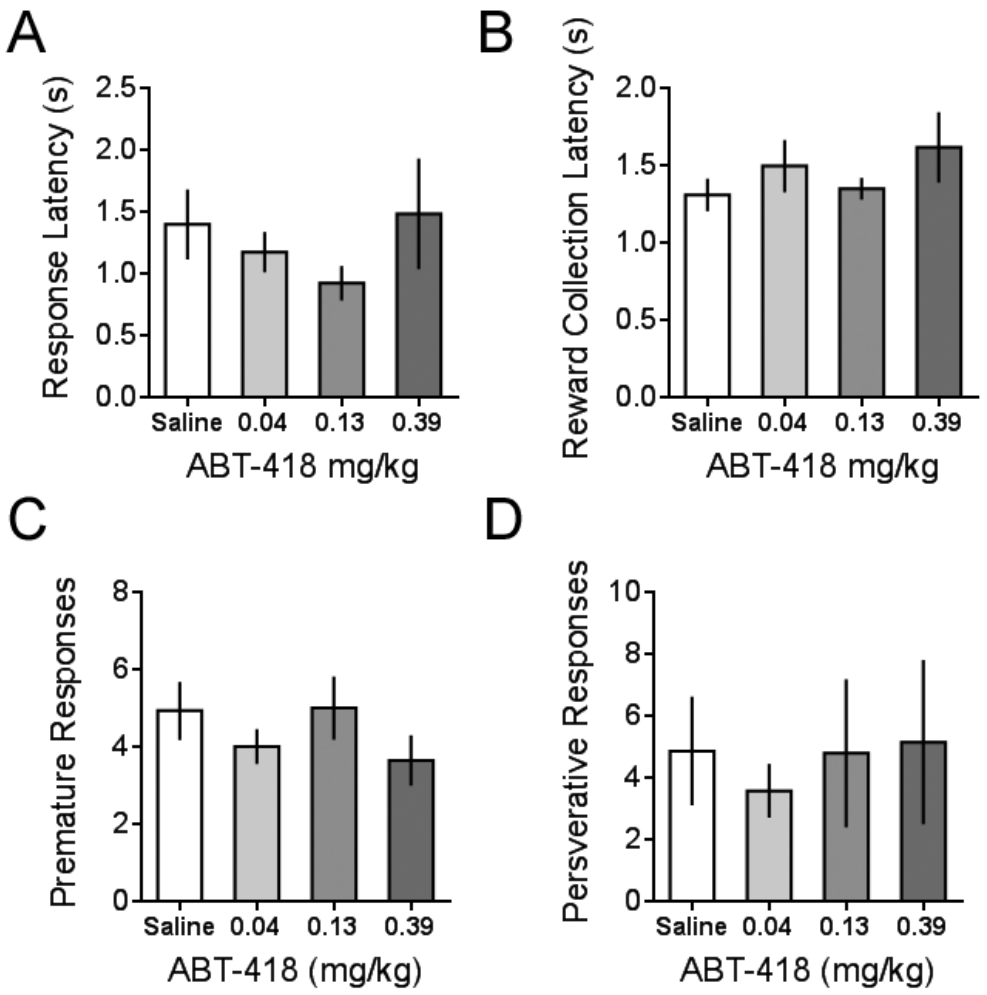


Figure 4.4.3. Sustained attention deficits of CHRNA7 null mice are reversed by β2nAChR agonists. (A) Omission and (B) accuracy following injections of ABT-418 in CHRNA7$^{-/-}$ mice. (C) Omissions and (D) accuracy over bins of 25 trials following administration of ABT-418. (E) cFos protein levels and (F) ERK1/2 phosphorylation following injection of ABT-418 (data are presented as mean ± SEM; *$P < 0.05$,**$P < 0.01$.

Figure 4.4.3. Sustained attention deficits of CHRNA7 null mice are reversed by β2nAChR agonists.

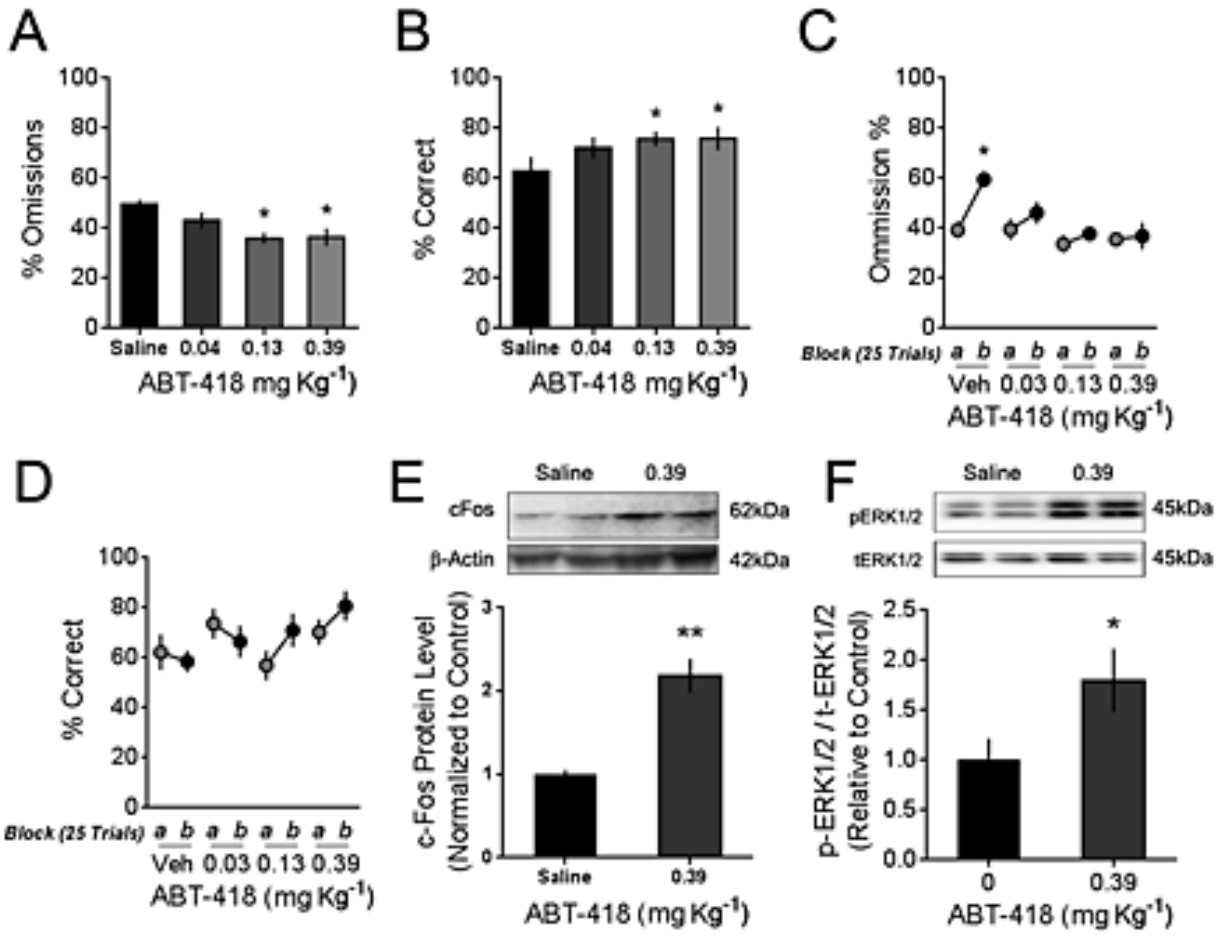


Figure 4.4.4. ABT-418 did not alter response patterns in CHRNA7 null mice. Premature responses (a), perseverative responses (b), response (c) and reward collection (d) latencies following ABT-418 injections in CHRNA7−/− mice. (Data are presented as mean ± SEM.).

Figure 4.4.4. ABT-418 did not alter response patterns in CHRNA7 null mice.

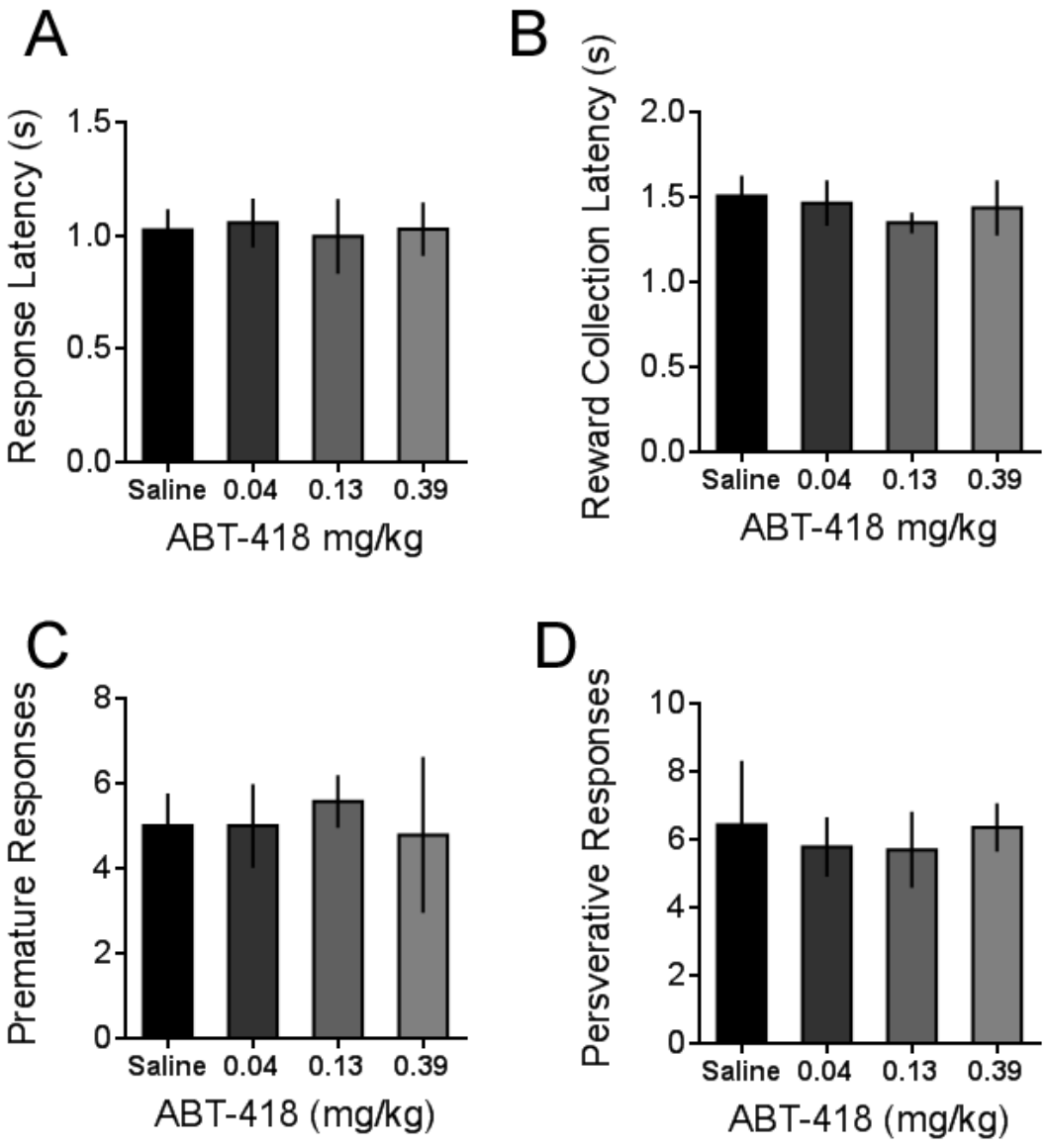
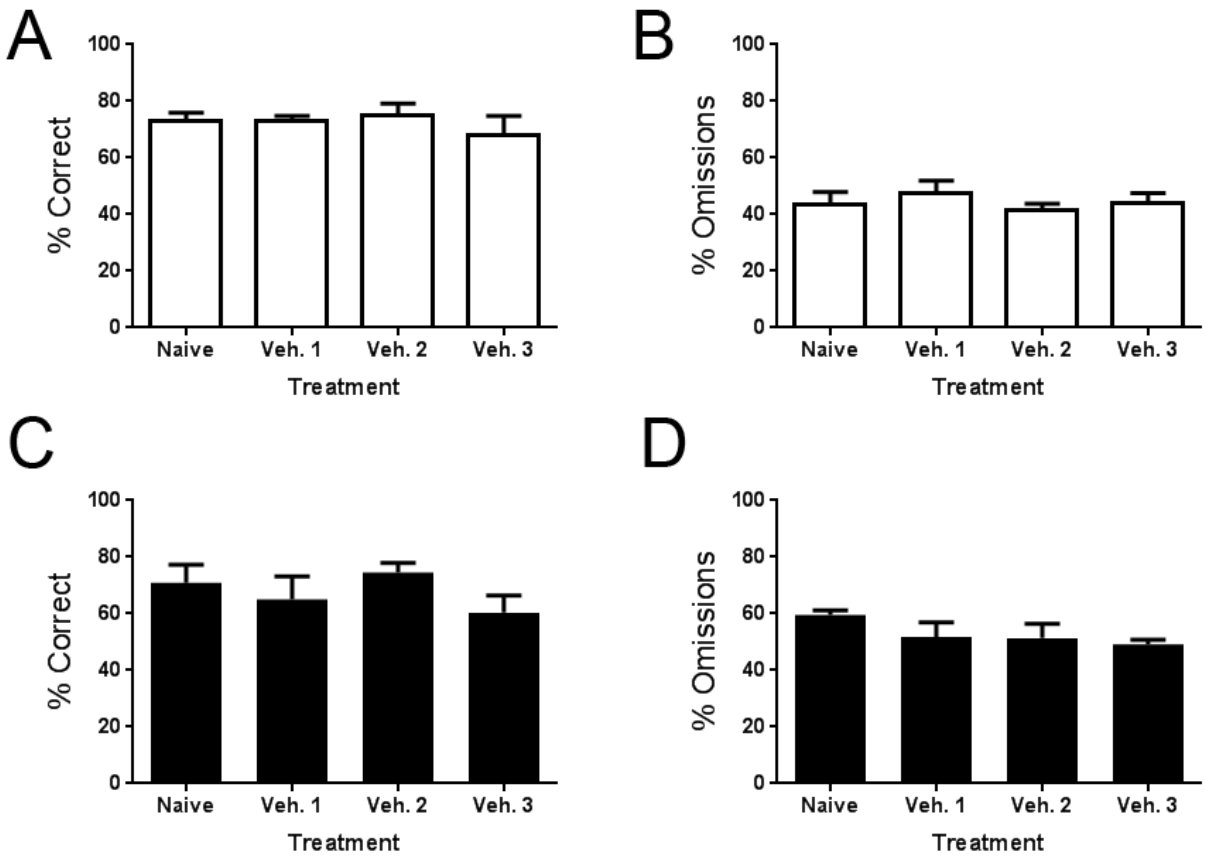


Figure 4.4.5. Performance of mice did not differ across all drug treatments. Evaluation of accuracy (a) and omissions (b) from vehicle treatments from all drug trials in wild-type mice. Evaluation of accuracy (c) and omissions (d) from vehicle treatments from all drug trials in wild-type mice CHRNA7−/− mice. (Data are presented as mean ± SEM.).

Figure 4.4.5. Performance of mice did not differ across all drug treatments.



4.6 Discussion

In this study, we demonstrated that the genetic elimination of the CHRNA7 gene disturbs sustained attentional performance, as measured by the 5-CSRT task, and that this deficit is reversed by administration of ABT-418, a β2nAChR agonist. CHRNA7−/− mice exhibited impaired performance (increased omission errors) during the second half of testing sessions of the 5-CSRT, suggestive of deficits in sustained attention, or vigilance. Increases in omission errors on the 5-CSRT task may reflect either decreased attentional processing or a lack of motivation (Robbins, 2002; Spinelli et al., 2004). However, given the normal performance of CHRNA7−/− mice on the food intake test, this phenotype is unlikely to represent a motivational issue, suggesting that the lack of α7nAChRs impairs the ability to maintain performance levels during the task. Pharmacological activation of α7nAChRs in WT mice by two distinct α7nAChR agonists, PHA-543,613 and PNU-282,987, in lower doses improved attentional performance but did not change sustained attention. These effects were specific to their actions on α7nAChRs, as these compounds were ineffective on CHRNA7−/− mice. Pharmacological activation of the β2nAChR by ABT-418 was able to reverse the sustained attention deficit in CHRNA7−/− mice, suggesting that the deficits observed in these mice can be rescued by β2nAChR signalling.

Post-mortem analysis of human patient samples has shown that ERK1/2 MAP kinase signalling is reduced in brains of schizophrenic patients (Yuan et al., 2010). Moreover, an inability to induce phosphorylation of ERK1/2 MAP kinases in the PFC is thought to underlie certain cognitive deficits in animal models of schizophrenia (Kamei et al., 2006). α7nAChR activation has been shown to induce phosphorylation of ERK1/2 MAP kinases both in vitro and in vivo (Bitner et al., 2007). Similar effects have been observed in second-generation antipsychotics (Lu et al., 2004). We showed that pharmacological activation of the α7nAChR by PHA-543,613 induced a dose response increase in both ERK1/2 phosphorylation levels and cFos in WT mice. These effects were not detected in CHRNA7−/− mice. Whether this biochemical correlation of α7nAChR activation relates to the biochemical and attentional deficits observed in schizophrenia is unknown. Interestingly, when CHRNA7−/− mice were treated with the β2nAChR agonist

ABT-418, both ERK1/2 phosphorylation and cFos protein levels were increased and were correlated with the reversal of the sustained attention deficits. Although it is currently unclear if the neurons that respond to ABT-418 and the α7nAChR drugs PHA-543,613 and PNU-282,987 are the same, one potential important implication of the behavioural data is that ABT-418 can reverse the sustained attention deficits due to abnormal CHRNA7 expression.

Interestingly, in WT mice, all α7nAChR targeting drugs used presented an inverted 'U'-shaped behavioural response. This is not uncommon with nicotinic signalling with similar responses observed across cognitive domains and even species (Picciotto, 2003; Olincy et al., 2006; Wallace et al., 2011a; Braida et al., 2013). Desensitization of the receptor is often suggested as a potential mechanism underlying this U-shaped behavioural response. Our data suggest that this may not be the case for α7nAChR activation, given that we see increases in the levels of phospho-ERK1/2 following administration of a high dose of α7nAChR agonist in WT mice, which results in poor performance on the 5-CSRT task. Interestingly, it has been proposed that overactivity of this second messenger signalling pathway can actually impair executive function and lead to distractibility (Birnbaum et al., 2004). We have also recently shown that increased cholinergic tone by overexpression of VAChT and increased cholinergic signalling in BAC ChAT-Chr2-EYFP mice disturbs attentional processing (Kolisnyk et al., 2013b).

The ability of nicotine to improve attention has been well documented in rodents (Young et al., 2004), non-human primates (Prendergast et al., 1998) and humans (Lawrence et al., 2002). A common technique to evaluate the role of α7nAChR signalling in attention has been to co-treat rodents with both nicotine and the α7nAChR antagonist MLA. These studies have, however, provided mixed results. Some investigators obtained evidence for α7nAChR signalling in the pro-attentive effects of nicotine (Hahn et al., 2011), and others failed to implicate α7nAChR signalling in nicotine-induced improvements in attention (Grottick and Higgins, 2000). Studies using AR-R17779, a full agonist of the α7nAChR, have failed to demonstrate pro-attentive effects of α7nAChR stimulation (Grottick and Higgins, 2000; Grottick et al., 2003; Hahn et al., 2003). This

compound, however, has also been shown to poorly penetrate the blood brain barrier (Mullen et al., 2000). On the other hand, R3487/MEM 3454, an α7nAChR agonist and 5-HT3 receptor antagonist, has been shown to improve measures of sustained attention in both rats (Rezvani et al., 2009) and macaque monkeys (Wallace et al., 2009). Our experiments utilized both PHA-543,613 and PNU-282,987, α7nAChR agonists, which have been previously reported to easily cross the blood brain barrier (Acker et al., 2008). Indeed, the biochemical activation reflected by increased cFos levels or phospho-ERK supports the contention that these drugs were able to activate the PFC in mice. Importantly, our data further support results from previous studies, suggesting that α7nAChR signalling has a role in sustained attention (Young et al., 2007), specifically characterized by increased omission errors on the 5-CSRT task in α7nAChR-null mice (Young et al., 2004).

Cholinergic transients in the PFC have been shown to be important for cue detection and attentional processing (Parikh et al., 2007). α7nAChR activation increases the duration of these transients 10–15-fold, and interestingly, this effect is lost when dopaminergic afferents to the PFC are eliminated, suggesting a complex interplay between neurotransmitter systems (Parikh et al., 2010).

An important role of nicotinic receptors in the CNS is to influence the release of other neurotransmitters. Nicotinic receptors have been shown to influence the release of glutamate (Gioanni et al., 1999), dopamine (Zhou et al., 2001), GABA (Alkondon et al., 1999), noradrenaline (Fu et al., 1998), 5-HT (Kenny et al., 2000) and ACh itself (Rowell and Winkler, 1984). Efflux of all of these neurotransmitters in PFC has been associated with performance on the 5-CSRT task (reviewed in Robbins, 2002). Electron microscopy studies point to ACh release potentially being auto-regulated by presynaptic α7nAChRs in the PFC (Duffy et al., 2009). On the other hand, post-synaptic β2nAChRs have been shown to be necessary and sufficient to regulate performance on the 5-CSRT task (Guillem et al., 2011; Poorthuis and Mansvelder, 2013). In addition to the possibility that α7 and β2 receptors can form functional heteromeric receptors (Liu et al., 2009; Moretti et al., 2014), our data reveal a complex interplay between these two receptors in regulating sustained attention. Given that our results suggest that activation

of β2nAChRs can bypass α7nAChRs, it is possible that activation of α7nAChRs could induce ACh release in the PFC, which would then activate post-synaptic β2nAChRs to regulate sustained attention. If this model is correct, it may explain the inconsistency amongst studies using non-selective nicotinic agonists and antagonists. Co-treatment with nicotine and methyllycaconitine, a α7nAChR antagonist, would still activate β2nAChRs and thus improve attentive processing. Therefore, these previous experiments would not exclude a role of α7nAChRs in attentional performance.

In conclusion, our data support a role for α7nAChRs in sustained attention and reveal an intricate relationship between distinct nicotinic receptors to regulate attentional performance. Our results indicate that activation of β2nAChRs can bypass attentional deficits due to α7nAChR deficiency, suggesting that β2nAChRs may be an important pharmacological target in cognitive dysfunctions in which impaired α7nAChRs have been implicated, such as schizophrenia and Alzheimer's disease (Parri et al., 2011).

4.7 Acknowledgements

This work was supported by CIHR (MOP 93651, 12600 and 89919), NSERC (402524-2013), the Weston Brain Institute, Brain Canada, Canadian Foundation for Innovation, ORF (Ontario Research Fund) and the Annie Dakens Research Fund Award from the Alzheimer's Society fellowship to B. K. M. A. A-O. gratefully acknowledges fellowship support from Kuwait University.

4.8 References

Acker BA, Jacobsen EJ, Rogers BN, Wishka DG, Reitz SC, Piotrowski DW et al. (2008). Discovery of N-[(3R,5R)-1-azabicyclo[3.2.1]oct-3-yl]furo[2,3-c]pyridine-5-carboxamide as an agonist of the alpha7 nicotinic acetylcholine receptor: in vitro and in vivo activity. Bioorg Med Chem Lett 18: 3611–3615.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M et al. (2013a). The concise guide to pharmacology 2013/14: ligand-gated ion channels. Br J Pharmacol 170: 1582–1606.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M et al. (2013b). The concise guide to pharmacology 2013/14: transporters. Br J Pharmacol 170: 1706–1796.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M et al. (2013c). The concise guide to pharmacology 2013/14: enzymes. Br J Pharmacol 170:

Alkondon M, Pereira EF, Eisenberg HM, Albuquerque EX (1999). Choline and selective antagonists identify two subtypes of nicotinic acetylcholine receptors that modulate GABA release from CA1 interneurons in rat hippocampal slices. J Neurosci: Offic J Soc Neurosci 19: 2693–2705.

Azam L, Winzer-Serhan U, Leslie FM (2003). Co-expression of alpha7 and beta2 nicotinic acetylcholine receptor subunit mRNAs within rat brain cholinergic neurons. Neuroscience 119: 965–977.

Birnbaum SG, Yuan PX, Wang M, Vijayraghavan S, Bloom AK, Davis DJ et al. (2004). Protein kinase C overactivity impairs prefrontal cortical regulation of working memory. Science 306: 882–884.

Bitner RS, Bunnelle WH, Anderson DJ, Briggs CA, Buccafusco J, Curzon P et al. (2007). Broad-spectrum efficacy across cognitive domains by alpha7 nicotinic acetylcholine receptor agonism correlates with activation of ERK1/2 and CREB phosphorylation pathways. J Neurosci : Offic J Soc Neurosci 27: 10578–10587.

Braida D, Ponzoni L, Martucci R, Sparatore F, Gotti C, Sala M (2013). Role of neuronal nicotinic acetylcholine receptors (nAChRs) on learning and memory in zebrafish. Psychopharmacology (Berl) 231: 1975–1985.

Buckner RL (2004). Memory and executive function in aging and AD: multiple factors that cause decline and reserve factors that compensate. Neuron 44: 195–208.

Buschman TJ, Miller EK (2007). Top-down versus bottom-up control of attention in the prefrontal and posterior parietal cortices. Science 315: 1860–1862.

Chen L, Yamada K, Nabeshima T, Sokabe M (2006). alpha7 Nicotinic acetylcholine receptor as a target to rescue deficit in hippocampal LTP induction in beta-amyloid infused rats. Neuropharmacology 50: 254–268.

Corbetta M, Shulman GL (2002). Control of goal-directed and stimulus-driven attention in the brain. Nat Rev Neurosci 3: 201–215.

Duffy AM, Zhou P, Milner TA, Pickel VM (2009). Spatial and intracellular relationships between the alpha7 nicotinic acetylcholine receptor and the vesicular acetylcholine transporter in the prefrontal cortex of rat and mouse. Neuroscience 161: 1091–1103.

Elliott R (2003). Executive functions and their disorders. Br Med Bull 65: 49–59.

Fu Y, Matta SG, James TJ, Sharp BM (1998). Nicotine-induced norepinephrine release in the rat amygdala and hippocampus is mediated through brainstem nicotinic cholinergic receptors. J Pharmacol Exp Ther 284: 1188–1196.

Gioanni Y, Rougeot C, Clarke PB, Lepouse C, Thierry AM, Vidal C (1999). Nicotinic receptors in the rat prefrontal cortex: increase in glutamate release and facilitation of mediodorsal thalamo-cortical transmission. Eur J Neurosci 11: 18–30.

Grottick AJ, Haman M, Wyler R, Higgins GA (2003). Reversal of a vigilance decrement in the aged rat by subtype-selective nicotinic ligands. Neuropsychopharmacol: Offic Publication Am Coll Neuropsychopharmacol 28: 880–887.

Grottick AJ, Higgins GA (2000). Effect of subtype selective nicotinic compounds on attention as assessed by the five-choice serial reaction time task. Behav Brain Res 117: 197–208.

Guan ZZ, Zhang X, Blennow K, Nordberg A (1999). Decreased protein level of nicotinic receptor alpha7 subunit in the frontal cortex from schizophrenic brain. Neuroreport 10: 1779–1782.

Guillem K, Bloem B, Poorthuis RB, Loos M, Smit AB, Maskos U et al. (2011). Nicotinic acetylcholine receptor beta2 subunits in the medial prefrontal cortex control attention. Science 333: 888–891.

Guillozet-Bongaarts AL, Hyde TM, Dalley RA, Hawrylycz MJ, Henry A, Hof PR et al. (2014). Altered gene expression in the dorsolateral prefrontal cortex of individuals with schizophrenia. Mol Psychiatry 19: 1975–1985.

Guzman MS, De Jaeger X, Raulic S, Souza IA, Li AX, Schmid S et al. (2011). Elimination of the vesicular acetylcholine transporter in the striatum reveals regulation of behaviour by cholinergic-glutamatergic co-transmission. PLoS Biol 9: e1001194.

Hahn B, Sharples CG, Wonnacott S, Shoaib M, Stolerman IP (2003). Attentional effects of nicotinic agonists in rats. Neuropharmacology 44: 1054–1067.

Hahn B, Shoaib M, Stolerman IP (2011). Selective nicotinic receptor antagonists: effects on attention and nicotine-induced attentional enhancement. Psychopharmacology (Berl) 217: 75–82.

Hajos M, Hurst RS, Hoffmann WE, Krause M, Wall TM, Higdon NR et al. (2005). The selective alpha7 nicotinic acetylcholine receptor agonist PNU-282987 [N-[(3R)-1-azabicyclo[2.2.2]oct-3-yl]-4-chlorobenzamide hydrochloride] enhances GABAergic synaptic activity in brain slices and restores auditory gating deficits in anesthetized rats. J Pharmacol Exp Ther 312: 1213–1222.

Hoyle E, Genn RF, Fernandes C, Stolerman IP (2006). Impaired performance of alpha7 nicotinic receptor knockout mice in the five-choice serial reaction time task. Psychopharmacology 189: 211–223.

Kamei H, Nagai T, Nakano H, Togan Y, Takayanagi M, Takahashi K et al. (2006). Repeated methamphetamine treatment impairs recognition memory through a failure of novelty-induced ERK1/2 activation in the prefrontal cortex of mice. Biol Psychiatry 59: 75–84.

Kastner S, Ungerleider LG (2000). Mechanisms of visual attention in the human cortex. Annu Rev Neurosci 23: 315–341.

Kenny PJ, File SE, Neal MJ (2000). Evidence for a complex influence of nicotinic acetylcholine receptors on hippocampal serotonin release. J Neurochem 75: 2409–2414.

Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010). Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol 8: e1000412.

Kolisnyk B, Al-Onaizi MA, Hirata PH, Guzman MS, Nikolova S, Barbash S et al. (2013a). Forebrain deletion of the vesicular acetylcholine transporter results in deficits in executive function, metabolic, and RNA splicing abnormalities in the prefrontal cortex. J Neurosci: Offic J Soc Neurosci 33: 14908–14920.

Kolisnyk B, Guzman MS, Raulic S, Fan J, Magalhaes AC, Feng G et al. (2013b). ChAT-ChR2-EYFP mice have enhanced motor endurance but show deficits in attention and several additional cognitive domains. J Neurosci: Offic J Soc Neurosci 33: 10427–10438.

Lawrence NS, Ross TJ, Stein EA (2002). Cognitive mechanisms of nicotine on visual attention. Neuron 36: 539–548.

Liu Q, Huang Y, Xue F, Simard A, DeChon J, Li G et al. (2009). A novel nicotinic acetylcholine receptor subtype in basal forebrain cholinergic neurons with high sensitivity to amyloid peptides. J Neurosci : Offic J Soc Neurosci 29: 918–929.

Lu XH, Bradley RJ, Dwyer DS (2004). Olanzapine produces trophic effects in vitro and stimulates phosphorylation of Akt/PKB, ERK1/2, and the mitogen-activated protein kinase p38. Brain Res 1011: 58–68.

Martins-Silva C, De Jaeger X, Guzman MS, Lima RD, Santos MS, Kushmerick C et al. (2011). Novel strains of mice deficient for the vesicular acetylcholine transporter: insights on transcriptional regulation and control of locomotor behavior. PLoS One 6: e17611.

McGaughy J, Decker MW, Sarter M (1999a). Enhancement of sustained attention performance by the nicotinic acetylcholine receptor agonist ABT-418 in intact but not basal forebrain-lesioned rats. Psychopharmacology (Berl) 144: 175–182.

McGaughy J, Decker MW, Sarter M (1999b). Enhancement of sustained attention performance by the nicotinic acetylcholine receptor agonist ABT-418 in intact but not basal forebrain-lesioned rats. Psychopharmacology (Berl) 144: 175–182.

Mega MS, Cummings JL (1994). Frontal-subcortical circuits and neuropsychiatric disorders. J Neuropsychiatry Clin Neurosci 6: 358–370.

Mirnics K, Middleton FA, Marquez A, Lewis DA, Levitt P (2000). Molecular characterization of schizophrenia viewed by microarray analysis of gene expression in prefrontal cortex. Neuron 28: 53–67.

Moretti M, Zoli M, George AA, Lukas RJ, Pistillo F, Maskos U et al. (2014). The novel alpha7beta2-nicotinic acetylcholine receptor subtype is expressed in mouse and human basal forebrain: biochemical and pharmacological characterization. Mol Pharmacol 86: 306–317.

Mullen G, Napier J, Balestra M, DeCory T, Hale G, Macor J et al. (2000). (−)-Spiro[1-azabicyclo[2.2.2]octane-3,5′-oxazolidin-2′-one], a conformationally restricted analogue of acetylcholine, is a highly selective full agonist at the alpha 7 nicotinic acetylcholine receptor. J Med Chem 43: 4045–4050.

Olincy A, Harris JG, Johnson LL, Pender V, Kongs S, Allensworth D et al. (2006). Proof-of-concept trial of an alpha7 nicotinic agonist in schizophrenia. Arch Gen Psychiatry 63: 630–638.

Olincy A, Stevens KE (2007). Treating schizophrenia symptoms with an alpha7 nicotinic agonist, from mice to men. Biochem Pharmacol 74: 1192–1201.

Parikh V, Ji J, Decker MW, Sarter M (2010). Prefrontal beta2 subunit-containing and alpha7 nicotinic acetylcholine receptors differentially control glutamatergic and cholinergic signaling. J Neurosci : Offic J Soc Neurosci 30: 3518–3530.

Parikh V, Kozak R, Martinez V, Sarter M (2007). Prefrontal acetylcholine release controls cue detection on multiple timescales. Neuron 56: 141–154.

Parri HR, Hernandez CM, Dineley KT (2011). Research update: alpha7 nicotinic acetylcholine receptor mechanisms in Alzheimer's disease. Biochem Pharmacol 82: 931–942.

Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SP, Buneman OP et al. (2014). The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledgebase of drug targets and their ligands. Nucl. Acids Res. 42 (Database Issue): D1098–106.

Picciotto MR (2003). Nicotine as a modulator of behavior: beyond the inverted U. Trends Pharmacol Sci 24: 493–499.

Pichat P, Bergis OE, Terranova JP, Urani A, Duarte C, Santucci V et al. (2007). SSR180711, a novel selective alpha7 nicotinic receptor partial agonist: (II) efficacy in experimental models predictive of activity against cognitive symptoms of schizophrenia. Neuropsychopharmacol: Offic Publication Am Coll Neuropsychopharmacol 32: 17–34.

Poorthuis RB, Mansvelder HD (2013). Nicotinic acetylcholine receptors controlling attention: behavior, circuits and sensitivity to disruption by nicotine. Biochem Pharmacol 86: 1089–1098.

Prendergast MA, Jackson WJ, Terry AV Jr, Decker MW, Arneric SP, Buccafusco JJ (1998). Central nicotinic receptor agonists ABT-418, ABT-089, and (−)-nicotine reduce distractibility in adult monkeys. Psychopharmacology (Berl) 136: 50–58.

Rezvani AH, Kholdebarin E, Brucato FH, Callahan PM, Lowe DA, Levin ED (2009). Effect of R3487/MEM3454, a novel nicotinic alpha7 receptor partial agonist and 5-HT3 antagonist on sustained attention in rats. Prog Neuropsychopharmacol Biol Psychiatry 33: 269–275.

Robbins TW (2002). The 5-choice serial reaction time task: behavioural pharmacology and functional neurochemistry. Psychopharmacology (Berl) 163: 362–380.

Romberg C, Mattson MP, Mughal MR, Bussey TJ, Saksida LM (2011). Impaired attention in the 3xTgAD mouse model of Alzheimer's disease: rescue by donepezil (Aricept). J Neurosci : Offic J Soc Neurosci 31: 3500–3507.

Rowell PP, Winkler DL (1984). Nicotinic stimulation of [3H]acetylcholine release from mouse cerebral cortical synaptosomes. J Neurochem 43: 1593–1598.

Semenova S, Markou A (2007). The effects of the mGluR5 antagonist MPEP and the mGluR2/3 antagonist LY341495 on rats' performance in the 5-choice serial reaction time task. Neuropharmacology 52: 863–872.

Spinelli S, Pennanen L, Dettling AC, Feldon J, Higgins GA, Pryce CR (2004). Performance of the marmoset monkey on computerized tasks of attention and working memory. Brain Res Cogn Brain Res 19: 123–137.

Vicens P, Ribes D, Heredia L, Torrente M, Domingo JL (2013). Motor and anxiety effects of PNU-282987, an alpha7 nicotinic receptor agonist, and stress in an animal model of Alzheimer's disease. Curr Alzheimer Res 10: 516–523.

Wallace TL, Callahan PM, Tehim A, Bertrand D, Tombaugh G, Wang S et al. (2011a). RG3487, a novel nicotinic alpha7 receptor partial agonist, improves cognition and sensorimotor gating in rodents. J Pharmacol Exp Ther 336: 242–253.

Wallace TL, Chiu G, Dao H, Lowe DA, Porter R, Santarelli L (2009). R3487/MEM 3454, a novel nicotinic alpha 7 receptor partial agonist, improves attention and working memory performance in cynomolgus macaques. Biochem Pharmacol 78: 912–912.

Wallace TL, Porter RH (2011b). Targeting the nicotinic alpha7 acetylcholine receptor to enhance cognition in disease. Biochem Pharmacol 82: 891–903.

Wang HY, Lee DH, Davis CB, Shank RP (2000). Amyloid peptide Abeta(1-42) binds selectively and with picomolar affinity to alpha7 nicotinic acetylcholine receptors. J Neurochem 75: 1155–1161.

Weinberger DR, Berman KF, Zec RF (1986). Physiologic dysfunction of dorsolateral prefrontal cortex in schizophrenia. I. Regional cerebral blood flow evidence. Arch Gen Psychiatry 43: 114–124.

Xu J, Pato MT, Torre CD, Medeiros H, Carvalho C, Basile VS et al. (2001). Evidence for linkage disequilibrium between the alpha 7-nicotinic receptor gene (CHRNA7) locus and schizophrenia in Azorean families. Am J Med Genet 105: 669–674.

Young JW, Crawford N, Kelly JS, Kerr LE, Marston HM, Spratt C et al. (2007). Impaired attention is central to the cognitive deficits observed in alpha 7 deficient mice. Eur Neuropsychopharmacol: Journal Eur Coll Neuropsychopharmacol 17: 145–155.

Young JW, Finlayson K, Spratt C, Marston HM, Crawford N, Kelly JS et al. (2004). Nicotine improves sustained attention in mice: evidence for involvement of the alpha7 nicotinic acetylcholine receptor. Neuropsychopharmacol: Offic Publication Am Coll Neuropsychopharmacol 29: 891–900.

Young JW, Meves JM, Tarantino IS, Caldwell S, Geyer MA (2011). Delayed procedural learning in alpha7-nicotinic acetylcholine receptor knockout mice. Genes Brain Behav 10: 720–733.

Yu WF, Guan ZZ, Nordberg A (2007). Postnatal upregulation of alpha4 and alpha3 nicotinic receptor subunits in the brain of alpha7 nicotinic receptor-deficient mice. Neuroscience 146: 1618–1628.

Yuan P, Zhou R, Wang Y, Li X, Li J, Chen G et al. (2010). Altered levels of extracellular signal-regulated kinase signaling proteins in postmortem frontal cortex of individuals with mood disorders and schizophrenia. J Affect Disord 124: 164–169.

Zammit S, Spurlock G, Williams H, Norton N, Williams N, O'Donovan MC et al. (2007). Genotype effects of CHRNA7, CNR1 and COMT in schizophrenia: interactions with tobacco and cannabis use. Br J Psychiatry: J Ment Sci 191: 402–407.

Zhou FM, Liang Y, Dani JA (2001). Endogenous nicotinic cholinergic activity regulates dopamine release in the striatum. Nat Neurosci 4: 1224–1229.

Chapter 5

Cholinergic surveillance over hippocampal RNA metabolism and Alzheimer's-like pathology

This is a pre-copyedited, author-produced version of an article accepted for publication in *Cerebral Cortex* following peer review.

5.1 Chapter Summary

The relationship between long-term cholinergic dysfunction and risk of developing dementia is poorly understood. Here we used mice with deletion of the vesicular acetylcholine transporter (VAChT) in the forebrain to model cholinergic abnormalities observed in dementia. Whole genome RNA-sequencing of hippocampal samples revealed that cholinergic failure causes changes in RNA metabolism. Remarkably, key transcripts related to Alzheimer's disease are affected. *BACE1* for instance, shows abnormal splicing caused by decreased expression of the splicing regulator hnRNPA2/B1. Resulting BACE1 overexpression leads to increased APP processing and accumulation of soluble $A\beta_{1\text{-}42}$. This is accompanied by age-related increases in GSK3 activation, tau hyper-phosphorylation, caspase-3 activation, decreased synaptic markers, increased neuronal death and deteriorating cognition. Pharmacological inhibition of GSK3 hyperactivation reversed deficits in synaptic markers and tau hyperphosphorylation induced by cholinergic dysfunction, indicating a key role for GSK3 in some of these pathological changes. Interestingly, in human brains there was a high correlation between decreased levels of VAChT and hnRNPA2/B1 levels with increased tau hyperphosphorylation. These results suggest that changes in RNA processing caused by cholinergic loss can facilitate Alzheimer's-like pathology in mice, providing a mechanism by which decreased cholinergic tone may increase risk of dementia.

5.2 Introduction

Alzheimer's disease (AD), the predominant form of dementia, is pathologically characterized by accumulation of amyloid plaques and neurofibrillary tangles that ultimately lead to neuronal death. One of the early alterations identified in AD-affected individuals with cognitive decline is a profound decrease in basal forebrain cholinergic neurons (Whitehouse et al., 1982), which gave rise to the cholinergic hypothesis of AD (Bartus et al., 1982). Accordingly, Alzheimer's Disease Neuroimaging Initiative data reveal atrophy of the basal forebrain in individuals with mild cognitive impairment (Grothe et al., 2014), and increased forebrain cholinergic atrophy in Alzheimer's-affected individuals (Grothe et al., 2013). Cholinergic dysfunction correlates with decreased hippocampal volume and pathology (Teipel et al., 2014). Furthermore, recent epidemiological data suggest that long-term use of drugs with anti-cholinergic activity by elderly individuals increases the future risk of dementia (Gray et al., 2015). These observations reveal an intimate, but poorly understood relationship, between cholinergic dysfunction and the pathological and cognitive deficits in AD. However, whether cholinergic malfunction has a causal role in increasing the risk of dementia or regulating pathology is unknown. Moreover, the causal and temporal relationships between cholinergic malfunctioning and long-term changes in hippocampal neurons in AD are still unclear.

To test the capacity of cholinergic tone to regulate long-term functions in target cells we examined the hippocampal transcriptome in genetically-modified mice with compromised hippocampal cholinergic tone. Using forebrain-specific deletion of the vesicular acetylcholine transporter (VAChT), a protein required for acetylcholine (ACh) release (de Castro et al., 2009; Prado et al., 2013), we unveil that long-term cholinergic deficiency causes global changes in gene expression and alternative splicing in the hippocampus. This leads to abnormal alternative splicing of BACE1 with consequent age-dependent changes in amyloid precursor protein (APP) processing, tau hyper-phosphorylation, hippocampal neuronal loss and cognitive decline. Comparative analyses in the AD brain enabled us to identify links between cholinergic deficiency and AD pathology, together supporting the notion that early cholinergic dysfunction may be

a pivotal step in AD pathology initiation and progression. Our data provide potential mechanisms to explain how cholinergic deficiency may facilitate pathology in AD.

5.3 Materials and Methods

5.3.1 Mouse lines

Generation of VAChTflox/flox mice was previously described (de Castro et al., 2009). VAChTNkx2.1-Cre-flox/flox mice were generated by crossing VAChTflox/flox (crossed for 5 generations with C57BL/6J) with the Nkx2.1-Cre mouse line (C57BL/6J-Tg(Nkx2-1- cre)2Sand/J), purchased from The Jackson Laboratory (JAX stock no. 008661). Unless otherwise stated, all control mice used were VAChTflox/flox littermates. All procedures were conducted in accordance with guidelines of the Canadian Council of Animal Care (CCAC) and in accordance with ARRIVE guidelines, at the University of Western Ontario with an approved institutional animal protocol (2008-127). Only male mice were used for all experiments.

5.3.2 RNA Sequencing

Mouse hippocampal tissue was rapidly dissected and total RNA was extracted from individual samples using the PureLink RNA Mini Kit (Ambion). 2 µg of total RNA were then sent to the Centre for Applied Genomics, The Hospital for Sick Children, where the cDNA library was prepared using the TruSeq Stranded Total Sample Preparation kit (Illumina) and run in a HiSeq 2500 platform with coverage of 200-250 million pair reads per lane. 5 animals were run per lane to obtain enough coverage for alternative splicing analysis (50 million pair reads per sample). The sequenced reads were aligned to the mouse genome using the TopHat program against the mouse genome in Ensembl (version EnsMart72) to enable quantification of splice junctions in addition to gene level measurements. Differential gene expression analysis was conducted using the Bioconductor DESeq package which accounts for the counts binomial distribution

(Anders and Huber, 2010). Datasets are available on ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-3897.

For human brains, we used the SQUARETM RNA library construction approach which utilizes different sets of 5'- and 3'-site specific primers to segregate all full-length transcripts into sub-pools defined by the selective nucleotides in the respective primers. Unlike traditional sequencing, which is based on the use of universal primers that produce a pool of fragmented RNA products for a given gene, we used 12 different sets of 3'-primers that complement all distinct di-nucleotides at transcript 3'-polyadenylation sites and enable separate sequencing of the corresponding intact RNA molecules for each of the primer sets through barcoding. This unprecedented depth of segregated brain RNA-Seq data was made publicly accessible by establishing a user-friendly website where the sequenced variants for any given brain-expressed transcript can be found (http://apainad.weebly.com/). Sequencing files were processed and analyzed for differential expression and functional enrichment.

RNA sequencing libraries made from the temporal gyrus samples yielded an average of 6.0*106 (STD=2.0*106) uniquely aligned 75 base pair (bp) single end reads, or approximately 7.0*107 (STD=1.8*107) total read counts when combining all 12 SQUARE fields for each sample. These reads were mapped against the GRGCh37/hg19 version of the Homo sapiens genome (http://genome.ucsc.edu/). Transcripts with more than 1 read per kilobase per million (RPKM) per SQUARE field were defined as being detected (Hebenstreit and Teichmann, 2011). An average of 6610±1367 genes per field were detected across the 12 fields (details in Supplementary Table 5.6.2). Expression criteria were set to RPKM>1 in at least one of the SQUARE fields, in at least 80% of the tested donor cohorts.

5.3.3 Immunofluorescence

Immunofluorescence experiments were performed as previously described (de Castro et al. 2009). Primary antibodies used were anti-Choline Transporter (CHT1; 1:200), which was kindly donated by Dr. R. Jane Rylett, University of Western Ontario, London, Ontario, anti-hnRNPA2/B1 (1:200 Santa Cruz Biotechnology Catalog no. sc-10035),

anti-Cleaved caspase-3 (1:500 Thermo Fisher Scientific, Catalog no. PA5-16335), anti-AT180 (1:1000 Thermo Fisher Scientific, Catalog no. EN-MN1040), anti-NeuN (1:200 PhoshphoSolutions, Catalog no. 583-FOX3), anti-GFAP, anti-PSD95. Sections were visualized by Zeiss LSM 510 Meta (Carl Zeiss, Oberkochen, Germany) confocal system (40x, 63x objectives, with an N.A. of 1.3 and 1.4, respectively) and by Leica TCS SP8 (Leica Microsystems Inc, Ontario, Canada) confocal system (63x objective, with an N.A. of 1.4), a 488-nm Ar laser and 633-nm HeNe laser were used for excitation of fluorophores.

5.3.4 Western Blotting

Mouse hippocampi were collected, protein was isolated, and immunoblotting was performed as previously described using RIPA lysis buffer supplemented with protease and phosphatase inhibitors (Guzman et al., 2011). Band intensities were quantified using FluoroChemQ software (Thermo Fisher Scientific).

5.3.5 Gene Ontology Analysis

Gene ontology functional analysis was performed using the GOrilla software through the web application. Using the two-un-ranked lists method as described (Eden et al., 2009). KEGG pathway analysis was performed using the ClueGO plug-in of Cytoscape (Bindea et al., 2009).

5.3.6 RNA Binding Protein Analysis

To predict potential RNA-binding proteins that may be implicated in the observed changes in alternative splicing, alternatively spliced sequences were run through the RBPmap software (Paz et al., 2014) to detect potentially altered RNA binding proteins. The list of RNA binding proteins that were suggested by the software were then run through the Allen Brain Atlas (http://mouse.brain-map.org/) in order to ensure that they were expressed in the murine hippocampus. All RNA binding proteins not expressed in the hippocampus were excluded.

5.3.7 qPCR

To measure mRNA expression, total RNA was extracted from freshly dissected hippocampal tissue, using the Aurum Total RNA for fatty and fibrous tissue kit (Bio-Rad) according to the manufacturer's instructions. cDNA synthesis and qPCR analysis were performed as previously described (Guzman et al., 2011). For alternative splicing experiments, the alternative exon levels were normalized to a constitutively expressed exon from the same gene.

5.3.8 Primary Neuronal Cultures

Primary mouse hippocampal neurons were produced from E16 embryos as previously described (Ostapchenko et al., 2013). Neurons were cultured for 15 days. Knockdown of hnRNPA2/B1 from the cultured neurons was achieved by treatment with a shRNA, as previously described (Berson et al., 2012). A separate set of cultured neurons was treated with 10μM of carbachol, 10μM of carbachol and 100μM Atropine, or 100μM Atropine alone for 48 hours.

5.3.9 APP Processing

Biochemical analysis of the processing of APP was performed as previously described (Dewachter et al., 2002). Forebrains from VAChT deficient and control mice were homogenized in 50mM Tris-HCl (pH 8.5), samples were then ultracentrifuge at 135,000g for 1 hour at 4oC, and the supernatant was collected and analyzed by Western blotting and ELISA. The pellet was re-suspended and ultracentrifuged again and diluted in 8M guanidine HCl to obtain the insoluble fraction for ELISA analysis.

5.3.10 ELISA

Murine β-amyloid was measured from the hippocampal homogenate using the Wako Human/Rat (Mouse) β-Amyloid (42) ELISA High-Sensitive Kit (Catalog Number: 292-64501). The ELISA assay was performed according to the manufacturer's protocol.

5.3.11 Congo Red Staining

Congo red staining was performed as previously described (Thompson and Walker, 2015), using a Congo-Red solution (Sigma C-6277) in 100% ethanol.

5.3.12 Silver staining

Assessment of argyrophilic cells in the hippocampus was done by using NeuroSilverTM staining kit II (FD NeuroTechnologies, Inc., Baltimore, MD), which provides detection of degenerating neurons, including neuronal somata, axons, and terminals.

5.3.13 Estimation of Hippocampal Volume

NeuN immunohistochemistry was performed in order to estimate the volume of and number of neurons of hippocampal regions CA1, CA3, and the dentate gyrus (DG) as described (Beauquis et al., 2014). Briefly, tissue sections were stained with mouse monoclonal anti-NeuN (1:500 PhosphoSolutions, Catalog no. 583-FOX3), using the ABC kit (Vector Laboratories) and developed with 2 mM diaminobenzidine (Sigma, USA) and 0.5 mMH2O2 in 0.1 M Tris buffer. The total number of NeuN (T) cells in the various hippocampal regions was estimated using the following formula: $T=(N*V)/t$, where N is the cell density, V is the volume of the structure, and t is the thickness of the section.

5.3.14 GSK3 Inhibition

To inhibit GSK3 in VAChTNkx2.1-Cre-flox/flox mice, a cohort of aged animals (12 months old, n=5 AR-A014418 treated, n=4 saline treated) were implanted with Alzet micro-osmotic pumps (Model 1004; DURECT Corp, Cupertino, Calif). The pumps were implanted subcutaneously in the intra-scapular region of each mouse. The reservoir of each pump was preloaded with 96 µL of either sterile saline solution or the GSK3 inhibitor AR-A014418. The pumps administered 5 mg/kg/d of AR-A014418, a dose shown to produce a significant inhibition of GSK3 in vivo (Ly et al., 2013). During the

implantation procedure, mice were anesthetized with ketamine (100 mg/kg) and xylazine (15 mg/kg). Drug treatment lasted for 28 days.

5.3.15 Morris Water Maze

The spatial version of the Morris water maze (MWM) was conducted as described previously to investigate spatial memory (Kolisnyk et al., 2013b; Martyn et al., 2012; Vorhees and Williams, 2006). Briefly, animals were given four training trials a day (90 s each) for 4 d, with a 15 minute inter-trial interval. If the mice did not find the platform after 90 s during the learning phase, they were gently directed to the platform. On the fifth day, memory was assessed via a probe trial (60 s), during which the platform is removed and time spent in the target quadrant is measured. The task was performed in a 1.5-m-diameter pool with 25°C water. The platform was submerged 1 cm below the surface of the water, and spatial cues (posters, streamers, and plastic props) were distributed around the pool. Sessions were recorded and analyzed using the ANY-Maze Software.

The classification of search strategies mice employed during training was defined as previously described (Garthe et al., 2009). An experimenter blind to genotypes scored search strategies as follows: (1) thigmotaxis, characterized by maintaining close proximity to the wall (>70% trial within 10-cm of wall); (2) random search, illustrated by global swimming with no classified strategy; (3) scanning, characterized by a preference for the central pool area (>50% trial within 35-cm of pool center); (4) chaining, characterized by searching near the correct radial distance of the platform to the wall (>75% trial 20–50-cm from the pool center, <15% within 10-cm of wall, and <10% within 20-cm of pool center); (5) directed search, characterized by a preference for a passageway toward the platform or platform quadrant (>80% trial within a 50-cm-wide region from the start point to the platform); (6) focal search, characterized by a highly localized search near the platform (≥50% trial in a circular target zone with a 15-cm radius); (7) direct swim, characterized by a maintained heading toward the platform (Little to no deviation in path to reach platform from start point). Total block lengths were the sum of all blocks for one strategy and one mouse.

5.3.16 Protein Isolation from Human post-mortem brain tissue

Samples from parietal cortical tissues from age/ sex-matched controls (n = 6, 3 females and 3 males) and AD-affected individuals (n = 6, 3 females and 3 males) and information related to age and demographics have been previously published (Ostapchenko et al., 2013). The samples were homogenized in RIPA buffer supplemented with protease inhibitor cocktail (Calbiochem), and Western blotting was performed as described above.

5.3.17 Statistical analysis

Sigmastat 3.5 software was used for statistical analysis. Student's t-test was used for comparison between two experimental groups. Two-way ANOVA or two-way ANOVA with repeated measures (RM) were used when more than two groups were compared.

5.4 Results

5.4.1 Forebrain Cholinergic dysfunction modifies expression levels of hippocampal transcripts and alternative splicing

To determine the contribution of cholinergic tone to the regulation of hippocampal transcript levels, we used VAChTNkx2.1-Cre-flox/flox mice, a mouse line with selective deletion of the VAChT gene from forebrain regions, including the medial septum, which contains cholinergic neurons that project to the hippocampus. VAChT has been shown to be essential for ACh packaging and release (de Castro et al., 2009; Lima Rde et al., 2010; Prado et al., 2006). Non-biased whole genome transcriptome RNA-sequencing of hippocampal samples from three VAChT-deficient and four control mice yielded a total of 14,200 expressed genes. Comparative analysis revealed that 1,098 genes were differentially expressed in VAChTNkx2.1-Cre-flox/flox hippocampi compared to control mice (Figure 5.1.1A-B, FDR corrected $p < 0.05$). Of those, 763 genes were upregulated and 362 down-regulated in the transgenic mice. In addition, a linear regression analysis

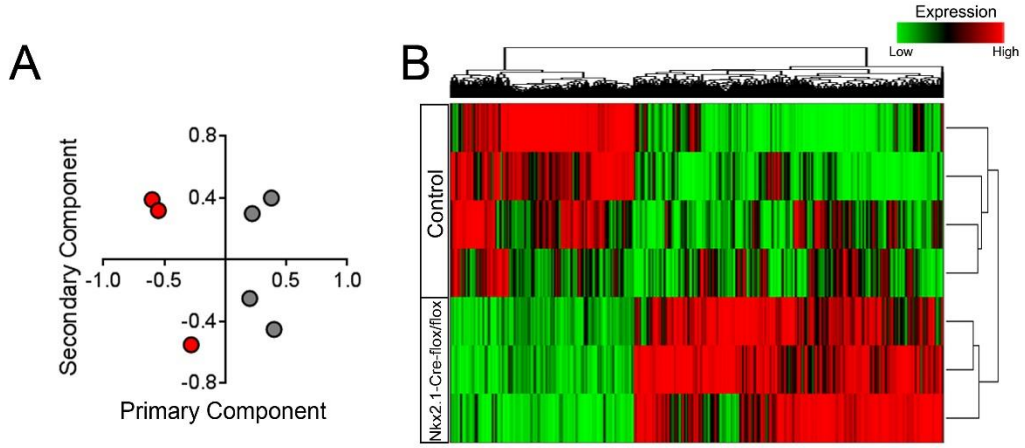
on reciprocal junction pairs detected roughly 4% of hippocampal transcripts in VAChTNkx2.1-Cre-flox/flox mice as alternatively spliced in high confidence as compared with control mice. Equal proportion of exon inclusion and exclusion events was observed; mainly events of cassette exons were detected (Figure 5.1.1C), suggesting widespread changes in several splicing regulation related pathways and/or cellular mechanisms (Soreq et al., 2014).

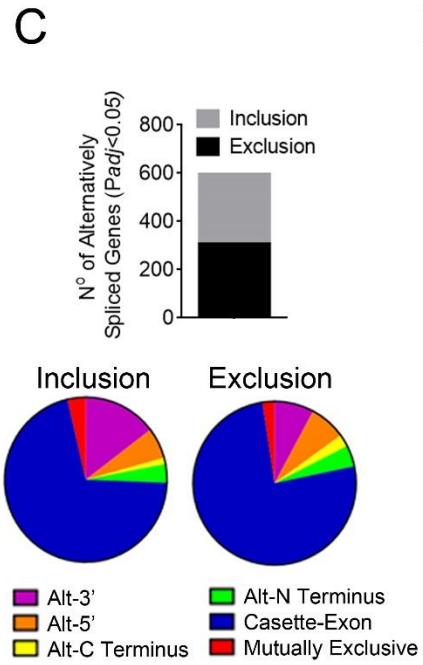
We interrogated these differentially expressed/spliced genes for involvement in neuronal function and AD-like pathology. A number of genes involved in critical pathways including PI3K-Akt signalling pathway [a regulator of neuronal vulnerability (Endo et al., 2006; Gary and Mattson, 2001)], spliceosome regulation and regulation of microtubule-based processes were identified using Gene Ontology (GO) KEGG pathway analysis (Figure 5.1.1D, Table 5.1). qPCR validation and correlation between changes observed in RNA-Seq and in an independent mouse cohort are shown in Fig. 5.1.2 for the different gene pathways and alternative splicing events. These results suggest that abnormal cholinergic signalling can effectively modulate several major gene pathways with potential to influence the function of target cells in the hippocampus.

We also performed small molecule RNA-Seq and additional miRNA microarray hybridization experiments, and observed limited changes in miRNA expression in the hippocampus of VAChT-deficient mice (Fig. 5.1.3). VAChTNkx2.1-Cre-flox/flox hippocampus showed a mature miRNA expression profile with only marginal differences from controls. Only 7 of 700 detectable miRNAs were differentially expressed, and of the 20 miRNAs most highly expressed in the hippocampus, comprising 82% of total counts, none were differentially expressed (Fig. 5.1.3). These findings point to alternative splicing and transcription, or changes in mRNA turnover, rather than miRNA, as potential main contributors to phenotypes in VAChT-deficient mice.

Figure 5.1.1. Forebrain deletion of VAChT induces alterations in hippocampal transcriptome. (A) Principal component analysis of transcripts from the hippocampi of control ($VAChT^{flox/flox}$; gray circles) and $VAChT^{NKx2.1\text{-}Cre\text{-}flox/flox}$ (red circles) mice. (B) Cluster analysis of differentially regulated transcripts in the hippocampus of $VAChT^{NKx2.1\text{-}Cre\text{-}flox/flox}$ (n=3) mice compared to controls (n=4). (C) Number of genes at indicated significance cut-off that were found to have an exon inclusion or exclusion event and summary of alternative splicing events. (D) List of genes from the PI3K-AKT pathway (grey shading), Spliceosome pathway (blue shading), Microtubule polymerization pathway (green shading) and other AD genes of interest (yellow shading), identified from KEGG pathway analysis of altered transcripts in $VAChT^{NKx2.1\text{-}Cre\text{-}flox/flox}$ mice. For each gene corresponding fold change, corrected statistical significance levels, and RNA expression change is also shown. Altered mRNA expression of these genes has been confirmed by qPCR (see Fig. 5.1.2).

Figure 5.1.1. Forebrain deletion of VAChT induces alterations in hippocampal transcriptome.





D

Gene	Log (Fold Change)	Corr. P Value	Group RNA Change
Bcar1	0.207	0.0296	
Col5a3	0.717	0.00801	
Fgfr1	0.219	0.0371	
Gng7	0.376	0.00110	
Itga4	0.178	0.0253	
Itga7	0.376	0.00645	
Lamb1	0.284	0.0108	Upregulated
Ngfr	0.841	0.00520	
Pck2	0.285	0.00497	
Prlr	1.638	0.000191	
Thbs3	0.628	6.96E-06	
Tnxb	0.490	0.00124	
Vegfc	0.431	0.0484	
Cdc40	0.238	0.00691	
Ddx39b	0.152	0.0495	
Hspa2	0.188	0.0476	
Snrnp70	0.205	0.0348	Upregulated
Srsf2	0.185	0.0141	
Srsf9	0.217	0.0279	
Wbp11	0.172	0.0321	
Apc	0.234	0.0438	
Camsap2	-0.539	0.00743	
Clasp1	-0.352	0.0103	
EBP4.1	-0.391	0.00924	
Lsp1	-0.535	0.0362	Alternatively Spliced: Exon Inclusion
Map1A	0.378	0.0135	
Mon2	0.453	0.0142	
Ncor1	0.950	0.0382	
Prkcz	0.600	0.0300	
Ralbp1	0.495	0.000741	
NOVA1	-0.317	0.0187	Downregulated
BACE1	0.570	0.0131	A.S. Inclusion

Figure 5.1.2. qPCR validation of RNA-Seq data. (A) qPCR validation and Pearson's correlation analysis on transcripts annotated to the spliceosome KEGG pathway quantified by RNA-Seq data from the hippocampus of VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ mice and controls [(r=0.8528, p=0.001), n=6 data are mean ± SEM.]. (B) qPCR validation and Pearson's correlation analysis with RNA-Seq data of transcripts annotated to the PI3k-AKT KEGG pathway [(r=0.8528, p=0.001). (C) qPCR validation and Pearson's correlation analysis of transcripts annotated to the microtubule polymerization pathway [(r=0.8528, p=0.001).

Figure 5.1.2. qPCR validation of RNA-Seq data.

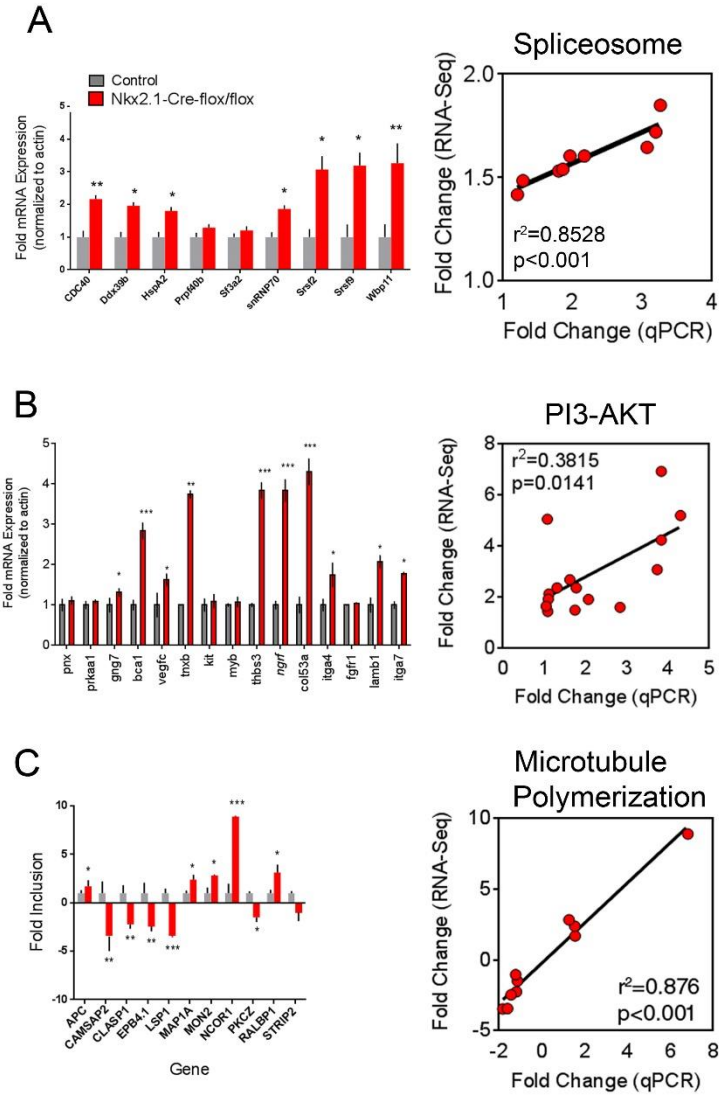


Figure 5.1.3. Global hippocampal expression of miRNA is not altered in VAChT deficient mice. (A) Small RNA sequencing from six VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice and five controls demonstrated miR-592-5p, miR-219-2-3 and 148a-3p as differentially expressed (DE) after FDR correction ($P<0.05$). (B) Principal Component Analysis (PCA) showed marginal separation of the two groups, suggesting that VAChT transcript removal does not induce a global change in small RNA expression. (C) Extent of absolute change was limited, as none of the top 20 miRNAs expressed in the hippocampus were modified, leaving 82% of hippocampal miRNAs unchanged. (D) Similar fractions of small RNA reads mapped to parts of the pre-miRNA molecules other than the mature-miRNA in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice and controls (E) Processing rate and/or efficacy might be affected, as a set of 7 seemingly unmodified miRNAs presented drastically reduced pre-miRNA levels when quantified by long RNA sequencing in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice.

Figure 5.1.3. Global hippocampal expression of miRNA is not altered in VAChT deficient mice.

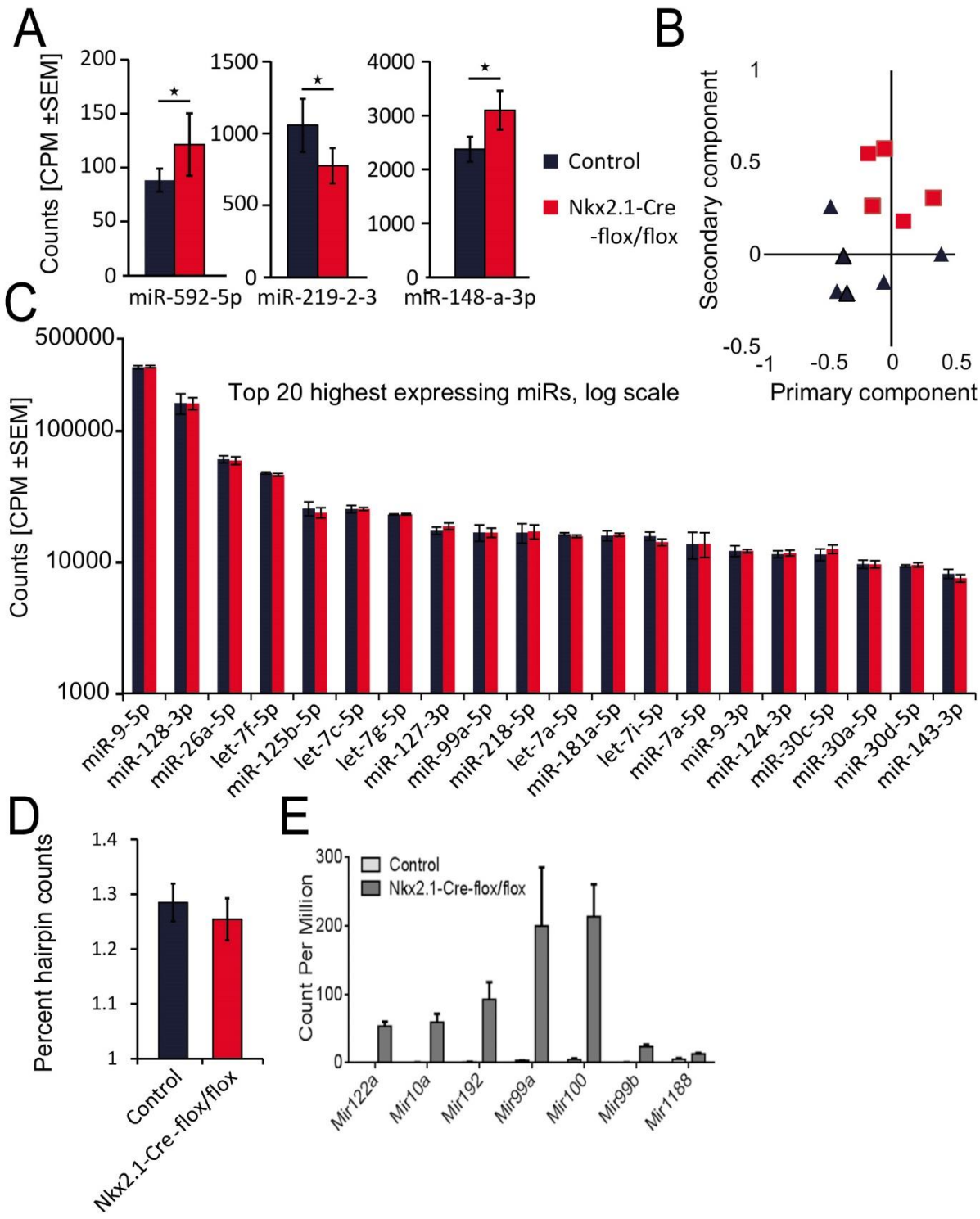


Table 5.1 AD Related Genes Identified in RNA-Seq Data. Genes in either the AKT pathway (grey shading), Spliceosome pathway (blue shading), or Regulation of Microtubule Polymerization (green shading) and role(s) in AD-like pathology.

Table 5.1 AD Related Genes Identified in RNA-Seq Data.

Gene	Link to AD
Bcar1	Linked to APP transcription (Seenundun and Robaire, 2007).
Col5a3	SNP linked to AD (Silver et al., 2012).
Fgfr1	Altered expression in aging (Walker et al., 1998).
Gng7	Associated with depression in the elderly (Schol-Gelok et al., 2010). Protein alterations in animal model of neurodegeneration (Karlsson et al., 2012).
Itga4	Upregulated in astrocytes in AD brain (Orre et al., 2014). Potential to bind Ab (Sabo et al., 1995).
Itga7	Potential to bind Ab (Sabo et al., 1995).
Kit	Mediates cell survival and apoptosis via AKT (Blume-Jensen et al., 1998)
Lamb1	SNP linked to AD (Taguchi et al., 2005)
Myb	Regulates neuronal apoptosis (Liu et al., 2004)
Ngfr	Up-regulated in AD brain (Scott et al., 1995) SNP linked to AD (Cozza et al., 2008)
Pck2	SNP linked to AD (Taguchi et al., 2005) Altered transcription in AD brain (Brooks et al., 2007)
Prkaa1	SNP linked to AD (Clarimon et al., 2009)
Prlr	Up-regulated in rat model of AD (Bakalash et al., 2011)
Pxn	Up-regulated in AD brain (Liang et al., 2012)
Thbs3	Altered transcription in AD brain (Kalback et al., 2004)
Tnxb	SNP linked to AD (Meda et al., 2012; Sherva et al., 2011)
Vegfc	Increased levels in CSF of AD patients (Tarkowski et al., 2002)
Cdc40	Linked to alternative splicing in neurodegeneration (Tollervey et al., 2011)
Ddx39b	SNP linked to Late-Onset-AD (Mohsen et al., 2015)
Hspa2	SNP linked to Late-Onset-AD (Broer et al., 2011; Clarimon et al., 2003)
Prpf40b	Implicated in human neurodegeneration (Passani et al., 2000)
Sf3a2	Altered in aging mouse brain(Meshorer and Soreq, 2002)
Snrnp70	Up-regulated and aggregated in AD brain (Bai et al., 2013)
Srsf2	Regulates splicing events in AD (Raj et al., 2014)

Srsf9	Regulates splicing events in AD (Qian and Liu, 2014)
Wbp11	Upregulated in accelerated aging murine model (Carter et al., 2005)
APC	Increased expression in AD brain (Leroy et al., 2001)
CAMSAP2	Altered protein levels in animal model of neurodegeneration (McGorum et al., 2015)
CLASP1	No known link to AD
EBP4.1	Interacts with APP protein in vivo (Bai et al., 2008)
LSP1	Epigenetic modifications in AD mouse model (Cong et al., 2014)
MAP1A	Upregulated in tau deficient mice (Ma et al., 2014) Binds soluble Aβ *in vitro (Clemmensen et al., 2012)*
MON2	No known link to AD
NCOR1	Protein expression altered by Aβ and tau (Hoerndli et al., 2007)
PKCZ	Altered activity in AD brain (Moore et al., 1998)
RALBP1	Transcription altered in AD brain (Zhang et al., 2015)
STRIP2	Decreased expression in neurodegeneration (Desplats et al., 2006)

5.4.2Cholinergic deficit triggers abnormal BACE1 alternative splicing and APP Processing

One of the detected abnormally alternatively spliced genes in our database was the protease BACE1 (Figure 5.2.1D), which is responsible for the cleavage of APP (Luo et al., 2001). The predicted alternative splicing event in VAChT-deficient mice is expected to increase expression of BACE1-501, the active protein isoform (Mowrer and Wolfe, 2008). qPCR analysis validated the predicted splicing event and demonstrated increased exon 3/4 inclusion for BACE1 (Figure 5.2.1A).

Bioinformatics analysis using the RBP-Map tool revealed an enrichment of binding sites for hnRNPA2/B1 in BACE1 mRNA. hnRNPA2/B1 is part of a family of RNA binding proteins that regulate pre-mRNA splicing, trafficking and maturation (Bekenstein and Soreq, 2013). Notably, AD-associated impairments in cholinergic signalling are accompanied by decreased expression of hnRNPA2/B1 protein in the AD cerebral cortex and in cholinergic impaired mice (Berson et al., 2012; Kolisnyk et al., 2013a). Correspondingly, the hippocampus of VAChTNkx2.1-Cre-flox/flox mice showed reduced hnRNPA2/B1 protein levels (Figure 5.2.1 B). We then investigated whether hnRNPA2/B1 regulates BACE1 splicing by exposing primary hippocampal cultured neurons to lentivirus carrying shRNA against hnRNPA2B1. Our results showed changes in BACE1 splicing similar to cholinergic deficiency (Figure 5.2.1C), directly implicating hnRNPA2/B1 in the regulation of BACE1 splicing. To test for the role of cholinergic signalling and the different cholinergic receptors in mediating this splicing event, we treated cultured hippocampal neurons with the cholinergic mimetic carbachol. This treatment was able to decrease the proportion of BACE1-501. This decrease was blocked by co-treatment with the muscarinic antagonist atropine (Figure 5.2.1D). These data implicate muscarinic receptors in the regulation of BACE1 splicing. This splicing event in BACE1 predicts an increase in the levels of the mature BACE1 protein and indeed, immunoblot analysis revealed a 2-fold increase of BACE1 levels in the hippocampus of VAChT-deficient mice (Figure 5.2.1E).

In late onset AD BACE1 expression is upregulated (Hebert et al., 2008) and it is thought to contribute to age-dependent progression in AD pathology (Ly et al., 2013). We therefore tested for changes in APP processing in VAChTNkx2.1-Cre-flox/flox mice. Aged VAChT-deficient mice (11-14 month old) displayed a modified pattern of Tris-soluble APP fragments (Figure 5.2.1 F), similar to that of mouse models with APP/PS1 mutations (Jankowsky et al., 2004; Oddo et al., 2003). In contrast, membrane-bound C-terminal fragments of APP (α and β CTFs), alterations of which can suggest impaired proteolytic processing of the protein [Reviewed in (Selkoe, 2000)], were similar in VAChT-deficient mice and controls (Figure 5.2.1 G). APP processing was not modified in aged Nkx2.1-Cre mice (Figure 5.2.2 A), suggesting that this effect is due to cholinergic dysfunction rather than to Cre expression.

We then assessed the levels of mouse amyloid peptides using an ELISA kit validated for both mouse and human Aβ peptides (Teich et al., 2013). The hippocampus of aged VAChTNkx2.1-Cre-flox/flox mice showed increased levels of soluble mouse A peptide compared to controls (Figure 5.2.1 H), reaching about one third of the levels of those found in aged 5XFAD mouse model of AD, which is one of the most aggressive models of AD amyloidosis. In comparison, insoluble amyloid peptide was undetectable in the brains of VAChT-deficient mice, whereas it was highly abundant in the 5XFAD mice (Figure 5.2.1 H). In addition, neither control nor VAChT-deficient mice displayed positive Congo red staining, unlike brain sections from 5XFAD mice, which exhibited numerous Congo red plaques (Figure 5.2.1 I). These data indicate that although VAChT-deficient mice show increased levels of soluble Aβ peptides, they do not seem to accumulate in extracellular amyloid plaques. Indeed, the murine amyloid peptide is much less prone to aggregation than human Aβ due to two amino acid changes (Jankowsky et al., 2007).

Figure 5.2.1 Disrupted APP processing and alternative splicing of BACE1 in cholinergic deficient mice. (A) qPCR analysis of alternative splicing events for BACE1 in the hippocampus of controls and (VAChT$^{flox/flox}$; gray bars) and VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ (red bars). Alternative exon levels are normalized to a constitutive exon from the same gene (n=6, data are mean ± SEM. **P<0.01). (B) Representative Western blot and quantification of hnRNPA2/B1 protein expression in the hippocampus of controls (VAChT$^{flox/flox}$; gray bars) and VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ (red bars) mice. hnRNPA2/B1 expression was normalized to actin (n=4, data are mean ± SEM. **P<0.01). (C) Quantification of the BACE1 alternative splicing in primary neuron cultures treated with hnRNPA2B1-shRNA (*P<0.05). (D) Quantification of the BACE1 alternative splicing in primary neuron cultures treated with 10mM Carbachol and Atropine (n=4, data are mean ± SEM. **P<0.05). (E) Representative Western blot and quantification of BACE1 protein levels in the hippocampus of controls (VAChT$^{flox/flox}$; gray bars) and VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ (red bars) mice. BACE1 expression was normalized to actin (n=3, data are mean ± SEM. *P<0.05). (F) Biochemical analysis and quantification of murine APP fragments in brain homogenates of aged expressed as a % [(Signal intensity of the fragment/signal intensity of full-length protein) x100]. 11-14 month old controls (VAChT$^{flox/flox}$; gray bars), VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ (red bars) and APPswe/PS1dE9 (green bars) hippocampal tissue extracts were resolved by Western blotting (data are mean ± SEM n=3). (G) Analysis of membrane-bound APP fragments in aged controls (VAChT$^{flox/flox}$; gray bars) and VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ (red bars) and APPswe/PS1dE9 (green bars) (data are mean ± SEM n=3). (H) Murine soluble and insoluble levels of Aβ42 in aged (11-14 months old) controls (VAChT$^{flox/flox}$; gray bars), VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ (red bars) and 5xFAD (black bars) measured by ELISA (n=4) (I) Congo red staining in the CA1 region of the hippocampus in aged (11-14 months old) controls, VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$, and 5xFAD mice. Arrowheads indicate positive-Congo red staining suggestive of amyloid plaques. (n=3, Scale bar, 100µm).

Figure 5.2.1 Disrupted APP processing and alternative splicing of BACE1 in cholinergic deficient mice.

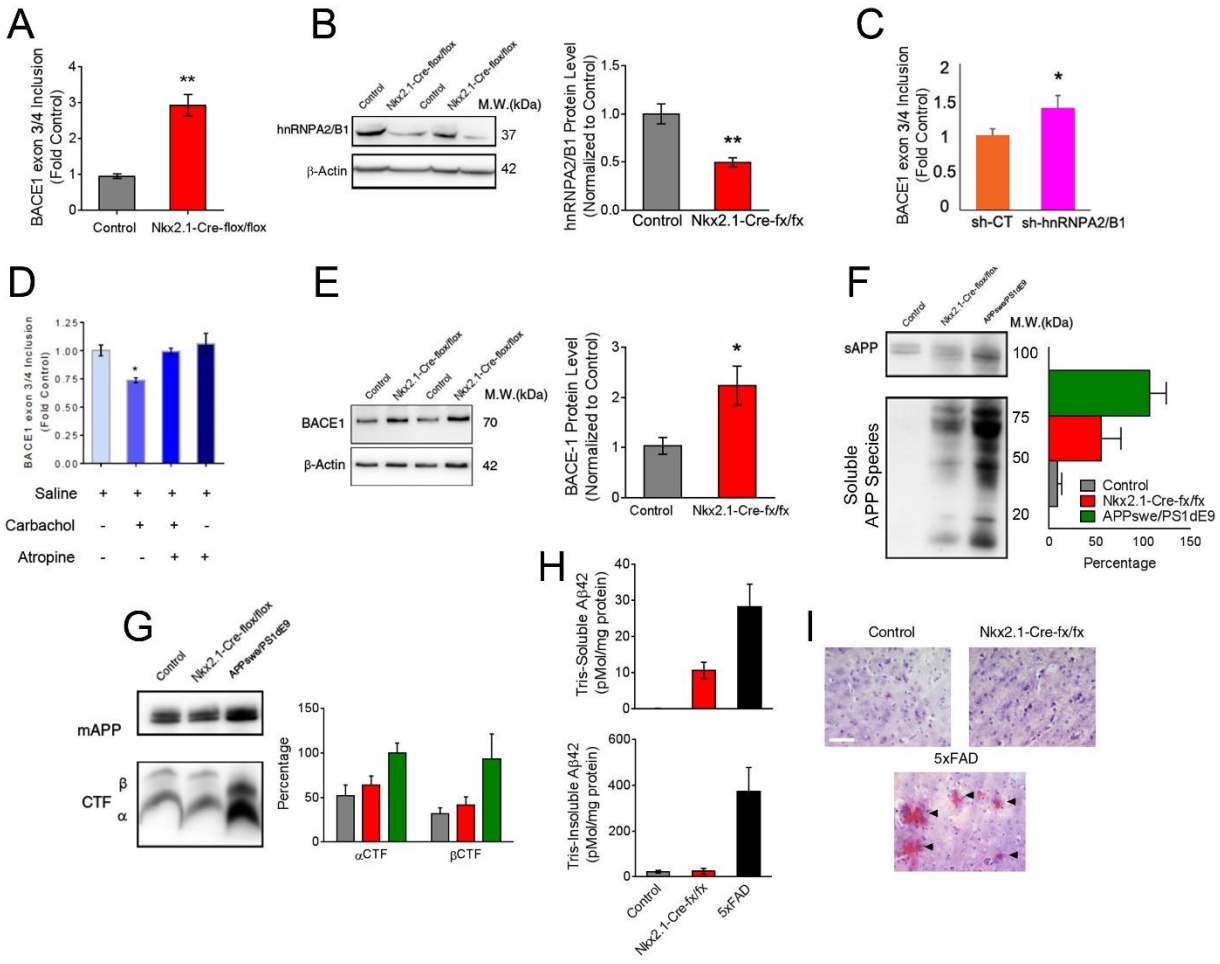
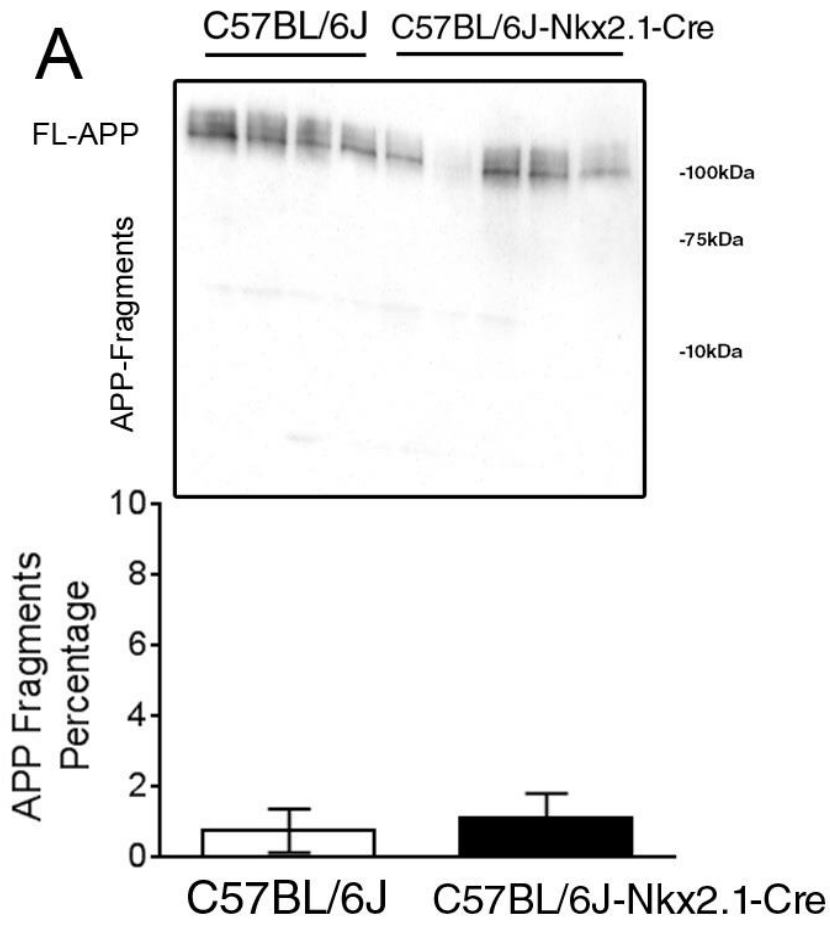


Figure 5.2.2 Absence of altered APP processing in aged (11-14 month old) C57/BJ6-Nkx2.1-Cre mice. (A) Western blot of APP processing from Tris-soluble fraction and quantification of APP fragments detected in aged C57BL/6J-Nkx2.1-Cre mice (11-14 month old) showing no significant differences compared to controls (n=4).(Data are mean +/- S.E.M.).

Figure 5.2.2 Absence of altered APP processing in aged (11-14 month old) C57/BJ6-Nkx2.1-Cre mice.



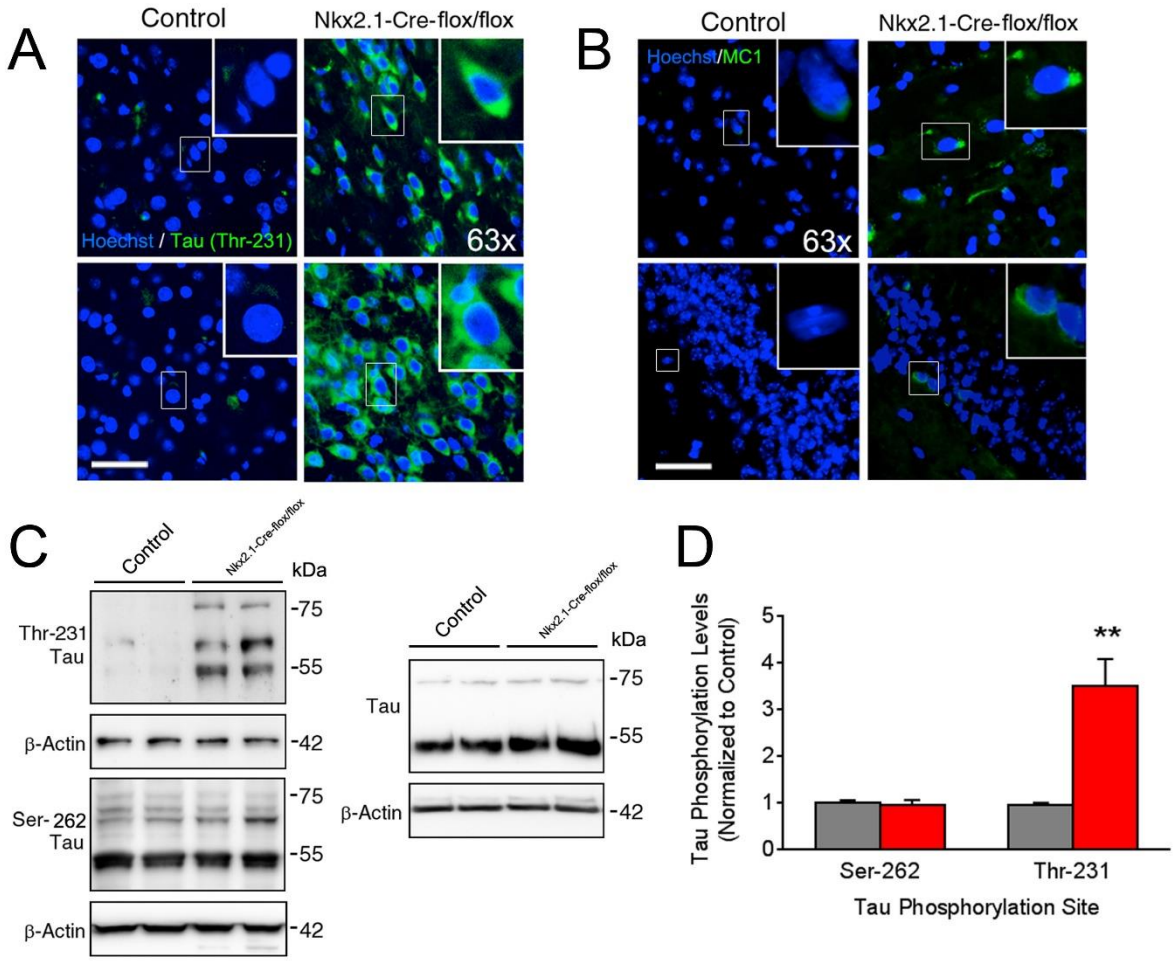
5.4.3 Cholinergic deficit leads to age-dependent hippocampal tauopathy

In AD, increased levels of soluble Aβ peptides are thought to precede abnormal phosphorylation of the microtubule associated protein tau (Iqbal et al., 2010). Previous reports suggested that cholinergic activity and tau phosphorylation might be inter-related (Hellstrom-Lindahl, 2000). Therefore, we used immunofluorescence to assess levels of the AT180 tau epitope (T231/S235) in the hippocampus of VAChT-deficient mice. This phosphorylation-dependent antibody specific to pT231 has been shown to label approximately 70% of paired helical filaments (PHF) in AD brains (Goedert et al., 1994). Phosphorylation at this epitope reduces the binding of tau to microtubules potentially increasing its toxicity (Lim et al., 2008). Immunofluorescence imaging revealed a robust increase of AT180 immunoreactivity in the hippocampus of VAChTNkx2.1-Cre-flox/flox mice compared to aged-matched controls (Figure 5.3A- 11-14 month-old mice). To test if the positive immunoreactivity of pTau in VAChT-deficient mice is associated with an induction of pathological tau, immunofluorescence with MC1 antibody was performed. Positive reactivity of conformation-dependent MC1 antibody depends on the proximity of N terminal (a.a. 7-9) and C-terminal (313-333) amino acid sequences of tau, which is one of the earliest alterations of tau in AD (Weaver et al., 2000; Wolozin et al., 1986). Immunostaining with MC1 revealed positive immunoreactivity in the hippocampus of aged cholinergic-deficient mice, but not in age-matched controls (Figure 5.3B).

In agreement with the immunofluorescence data, hippocampal extracts of VAChTNkx2.1-Cre-flox/flox mice showed approximately four-fold increases in pTau immunoreactive bands, including higher order oligomers detected with AT180, when compared to controls (Figure 5.3C and D). On the other hand, total tau and pTauS262 levels were unmodified in VAChT-deficient mice (Figure 5.3C and D). Taken together, our data indicate that deletion of hippocampal VAChT induces hyper-phosphorylation of tau and leads to tau pathological conformation as detected by MC1, both of which are consistently observed in AD. These data suggest the potential for neuronal toxicity due to cholinergic dysfunction.

Figure 5.3. Hippocampal cholinergic failure triggers tauopathy in an age-dependent manner. (A) Phosphorylated Tau levels monitored by immunolabeling with phosphorylation-dependent antibodies specific to pT231. Representative images of pT231 and Hoeschst labeling in the hippocampus of aged (11-14 month old) controls (left) and VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ (right) mice. (n=3, Scale bar, 100µm). (B) Representative images of MC1 and Hoeschst labeling in the CA1 region of the hippocampus of controls (left) and VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ (right) mice. (n=3, Scale bar, 100µm). (C) Western blot analysis of controls (VAChT$^{flox/flox}$) and VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ aged (11-14 month old) hippocampal samples for tau using phosphorylation-dependent anti-tau antibodies pT231, Ser 262 and for total Tau protein expression. (D) Quantification of Western blots. pT231, Ser 262, and total tau expression were normalized to actin (n= 4, data are mean ± SEM. **P<0.01).

Figure 5.3. Hippocampal cholinergic failure triggers tauopathy in an age-dependent manner.



5.4.4 Cholinergic deficiency exacerbates age-dependent neuronal vulnerability and impaired learning

Synaptic health is compromised in mouse models of AD and synaptic loss is a consistent finding in AD-affected individuals (Klein, 2006). To examine synaptic integrity we stained hippocampal sections with the synaptic marker PSD95. Aged VAChTNkx2.1-Cre-flox/flox mice displayed hippocampal decreases in PSD95-immunoreactivity, increased microglial activation and up-regulation of inflammatory markers, in comparison to age-matched controls, suggesting large-scale synaptic dysfunction in these mutants (Figure 5.4.1A-C). These observations predict neuronal vulnerability; therefore, we stained brain sections with silver, which accumulates in neurons that are more vulnerable to neurodegeneration (DeOlmos and Ingram, 1971). Aged VAChT-deficient mice presented intensified silver staining compared to controls; this increased silver staining was not observed in young VAChT-deficient mice (Figure 5.4.1D-E), suggesting that long-lasting decrease in cholinergic signalling may increase the vulnerability of hippocampal neurons. Parallel staining of hippocampal sections from aged 5XFAD mice compared to control mice performed as a positive control revealed similar increases in silver staining as that for VAChT-deficient mice (Figure 5.4.2).

Activated caspase-3, a marker of apoptosis, was augmented in young VAChTNkx2.1-Cre-flox/flox mice compared to controls (Figure 5.4.1F). However, aging significantly increased the number of activated caspase-positive cells in VAChT-deficient mice when compared to controls (Figure 5.4.1F). Also, young VAChT-deficient mice showed no alteration in the number of NeuN positive cells across all regions of the hippocampus (Figure 5.4.1G, Figure 5.4.2); while NeuN positive cells in CA1 and CA3 region, but not dentate gyrus were decreased in aged VAChT-deficient mice (Figure 5.4.1G, Figure 5.4.2), predicting functional implications for this aging-related hippocampal neuronal vulnerability. Thus, impaired cholinergic signalling induces global changes in transcript levels, followed by age-related exacerbation of synaptic and neuronal vulnerability.

To test whether long-lasting cholinergic failure may have age-dependent consequences in cognitive function, we subjected aged (11-14 months old) VAChT-deficient mice to

the MWM task. Young VAChT-deficient mice (3-6 months old) show little difference from controls in acquisition performance on the Morris Water Maze (MWM) task (Al-Onaizi et al., 2016). In contrast, aged VAChT-deficient mice took significantly longer and swam a greater distance than age-matched controls to find the platform across the four days of acquisition (Figure 5.4.1E-F). Furthermore, aged VAChT-deficient mice used distinct strategy preferences to find the platform, indicating that their deteriorated performance was due to modified learning capacities. Briefly, the analysis of search strategies used by each animal was based on a fixed set of criteria (Figure 5.4.1H). At a young age, both controls and VAChTNkx2.1-Cre-flox/flox mice predominantly used a more direct strategy to reach the platform (strategies 5/6/7, Figure 5.4.1I). In contrast, aged VAChT-deficient mice used random swimming predominantly as their strategy to acquire the task (strategy 2, Figure 5.4.1I), while aged control mice maintained the use of more direct strategies. Aged VAChT-deficient mice also exhibited deficits in the probe trial (Figure 5.4.1G). Taken together, these results suggest that long-term cholinergic deficiency in VAChTNkx2.1-Cre-flox/flox mice led to progressive loss of neurons in the hippocampus that worsened spatial information acquisition and cognitive functioning.

Figure 5.4.1. Hippocampal cholinergic failure leads to increased neuronal vulnerability and worsens cognitive functioning. (A) Immunofluorescence imaging showing PSD-95 protein levels in the hippocampus of aged controls or VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice and corresponding (B) Western blot analysis for PSD-95 protein levels in the hippocampus of VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice compared to controls ($t_{(6)}$=4.286, p=0.0052, n=4). (C) Immunofluorescence imaging and quantification showing CD-68 immunoreactivity in the hippocampus of aged VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice as well as levels of IL-1 transcripts as measured by qRT-PCR ($t_{(10)}$=2.312, p=0.0434, n=6) and IL-6 transcripts as measured by qRT-PCR ($t_{(10)}$=2.882, p=0.0204) (Data are mean +/- S.E.M., *P<0.05, n=6). (D) Representative images of silver staining in the CA1 region of young (3-6 months) and aged (11-14 months) mice. Scale bar, 100µm. (E) Quantification of silver stain-positive cells between young and aged hippocampi of controls (VAChT$^{flox/flox}$; gray bars) and VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ (red bars). (n=5, data are mean ± SEM. **P<0.01). (F) Representative immunofluorescence images for activated-caspase 3 labelling in the hippocampi of young (Top) and aged (Bottom) mice. Quantification of activated caspase-3 immunolabelling in young and aged hippocampi of controls (VAChT$^{flox/flox}$; gray bars) and VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ (red bars). (n= 3, data are mean ± SEM. *P<0.05, **P<0.01, Scale bar, 100µm). (G) Distribution of neuron-specific nuclear antigen (NeuN)-positive neurons in the CA1 region of the hippocampus in young (top) and aged (bottom) mice (Scale bar, 100µm). Quantitative comparison of the number of neurons labelled by NeuN in the CA1 region of the hippocampus in young (top) and aged (bottom) mice (n= 6, data are mean ± SEM. *P<0.05). (H) Representative examples of the 7 classified criteria to score the strategies mice used to perform in the MWM. Strategies are color coded. (I) Strategy plot reflecting the mean strategy-recruitment values for the first and fourth trials of each day for young (left) and aged (right) mice. Quantification comparison of total block length values of individual mice and their employed strategies over the course of 4-day training period. Grey bars represent control mice and red bars represent VAChT-deficient mice. (n= 8, data are mean ± SEM. *P<0.05 **P<0.01).

Figure 5.4.1. Hippocampal cholinergic failure leads to increased neuronal vulnerability and worsens cognitive functioning.

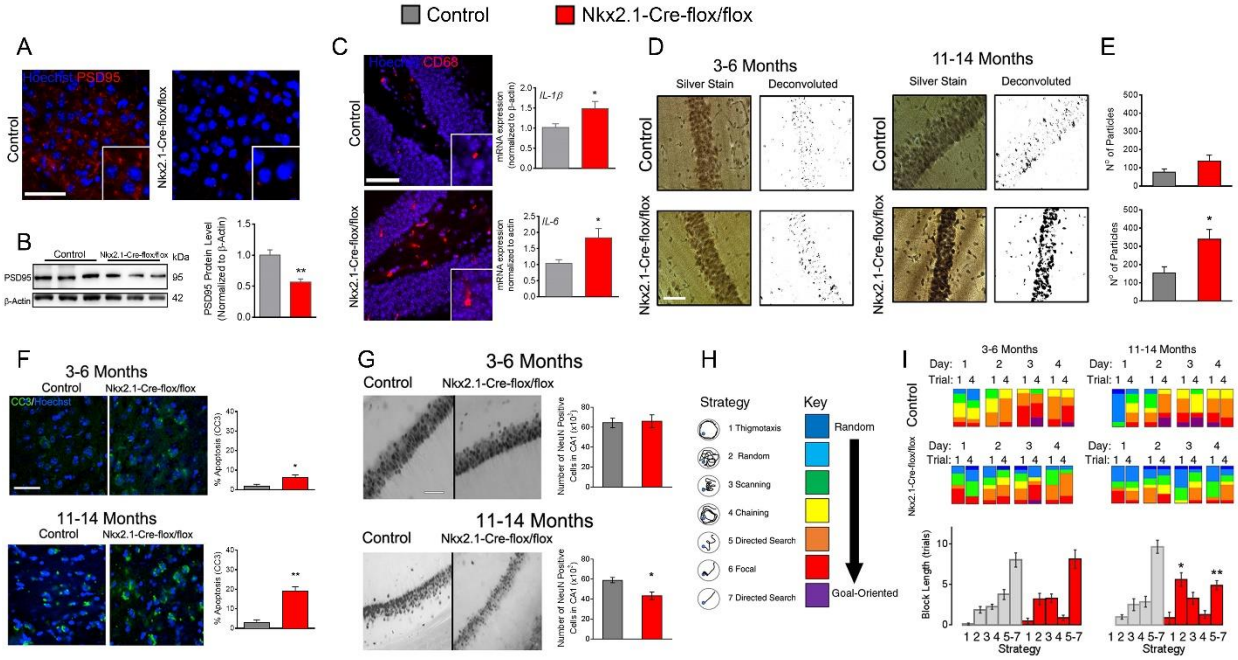
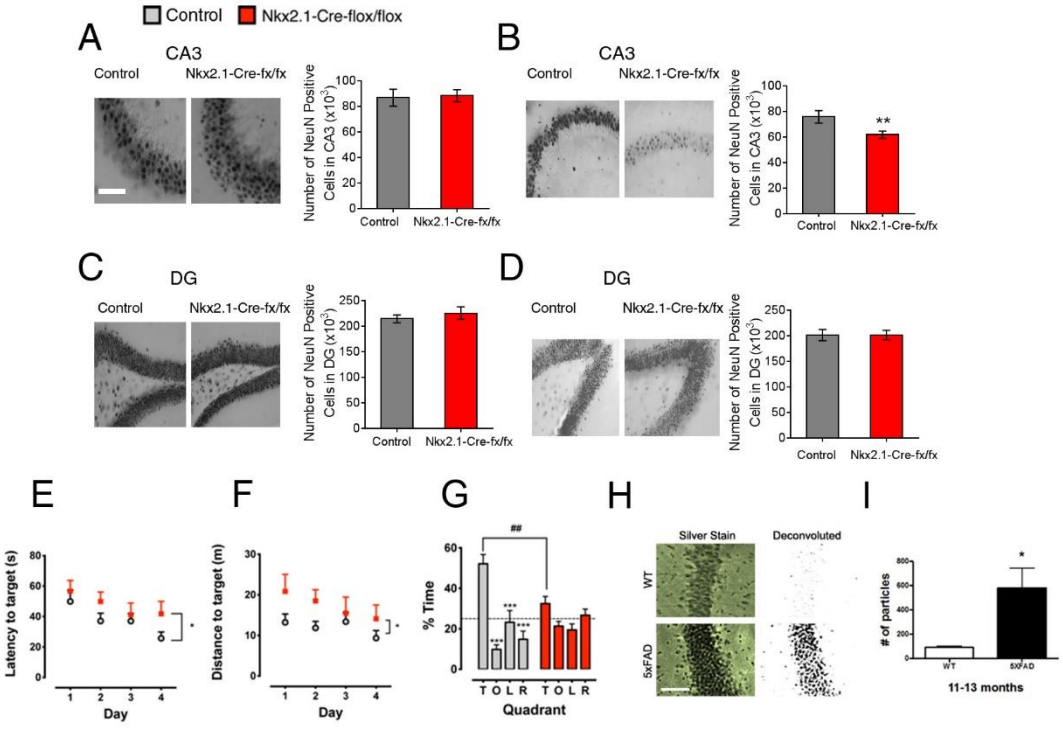


Figure 5.4.2 Estimation of neuronal volume in the CA3 and DG region of the hippocampus of young (3-6) and aged (11-14) month old cholinergic deficient mice as well as cognitive deficits in aged cholinergic deficient mice. (A) Young VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice show no change in number of NeuN positive cells in the CA3 ($t_{(18)}$=0.1894, p=0.8519), However in (B) there is a significant reduction in number of NeuN positive cells in the CA3 of aged VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice ($t_{(18)}$=2.454, p=0.0246). No change in the number of NeuN positive cells in the DG region was observed in either young (C) ($t_{(18)}$=0.7814, p=0.4447) or old (D) ($t_{(18)}$=0.01758, p=0.9862) VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice (Data are mean +/- S.E.M., **p<0.01, n=9). Significantly impaired latency (E) (RM-ANOVA, main effect of genotype $F_{(1,7)}$=6.359, p=0.0397), and distance to reach the platform (F) (RM-ANOVA, main effect of genotype $F_{(1,7)}$=7.845, p=0.0265) in aged (11-14 month old) VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice. (G) Aged (11-14 month old) VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice do not show a preference for the target platform during the probe trial portion of the MWM task (RM-ANOVA, main effect of interaction $F_{(3,21)}$=6.068, p<0.0038). (Data are mean +/- S.E.M., *p<0.05, **p<0.01, ***p<0.001, n=8). (H) Representative images of silver staining procedure from 11-14 month old wild-type and 5xFAD mice. (I) Quantification of percent silver stain area from 11-14 month old wild-type and 5xFAD mice (Data are mean +/- S.E.M., ***p<0.001, n=3). Scale bar 100 µm.

Figure 5.4.2 Estimation of neuronal volume in the CA3 and DG region of the hippocampus of young (3-6) and aged (11-14) month old cholinergic deficient mice as well as cognitive deficits in aged cholinergic deficient mice.



5.4.5 Cholinergic mediated age dependent pathology is partially mediated by GSK3 activation

In addition to APP processing and tau hyperphosphorylation, we observed other critical biochemical pathways altered in response to cholinergic deficiency, including aberrant GSK3 signalling, which has also been shown to play multiple roles in AD (Endo et al., 2006; Gary and Mattson, 2001). As several genes that regulate the PI3-AKT pathway were upregulated in VAChT-deficient mice (Fig. 5.1.1D, 5.1.2), we tested for dysregulation of PI3-AKT signalling pathway in these mice by evaluating the phosphorylation status of the AKT protein and its downstream target GSK3. AKT presented decreased phosphorylation at residue Ser473, with unmodified Thr308 phosphorylation, in VAChTNkx2.1-Cre-flox/flox hippocampus compared to controls (Figure 5.5.1A). Additionally, GSK3α/β tyrosine phosphorylation, which reflects activation of GSK3, was increased in these mutants (Figure 5.5.1B). Hence in addition to increased levels of proteins involved in AD pathology, these results suggest potential contributions of GSK3 activation in cholinergic-induced deficits.

To test the role of GSK3 in the abnormal hippocampal changes in cholinergic-deficient mice, we chronically treated a cohort of aged (11 months old) VAChTNKx2.1-Cre-flox/flox mice with the GSK3 inhibitor AR-A014418 (Figure 5.5.1C). After 28 days of treatment, we found that VAChT-deficient mice treated with AR-A014418 showed a significant decrease in GSK3 α and β tyrosine phosphorylation when compared to VAChTNKx2.1-Cre-flox/flox mice treated with saline (Figure 5.5.1D). Increased phosphorylation at Tyr residues 216 or 279 augments GSK3 activity (Hur and Zhou, 2010) and examining GSK3 phosphorylation at these residues has been used to determine the effectiveness of AR-A014418 (Carter et al., 2014; Yadav et al., 2014). We then assessed some of the key alterations detected in the hippocampus of aged VAChTNKx2.1-Cre-flox/flox mice. AR-A014418 treatment was able to significantly decrease tau T231 hyperphosphorylation and MC1-immunopositive tau in Western blots (Chai et al., 2011; Petry et al., 2014), by approximately 50% in VAChTNKx2.1-Cre-flox/flox mice when compared to saline VAChTNKx2.1-Cre-flox/flox mice. Total levels of tau where unchanged (Figure 5.5.1E). Immunofluorescence staining (Figure 5.5.1F)

also demonstrated reduced levels of T231 hyperphosphorylated tau in AR-A014418 treated mice. Compared to saline treated animals, AR-A014418 treatment was able to significantly increase levels of PSD-95 protein (Figure 5.5.1G).

Interestingly, we observed no changes in protein levels of BACE1 following AR-A014418 treatment in aged VAChTNKx2.1-Cre-flox/flox mice (Fig. 5.5.2A). Furthermore AR-A014418 treatment did not alter the alternative splicing event in the BACE1 gene (Figure 5.5.2A). Together, these data suggest that the hnRNPA2/B1-mediated alternative splicing and subsequent increase in BACE1 protein level are not mediated by GSK3 activation. Surprisingly, despite reduced levels of hyperphosphorylated tau, 1-month AR-A014418 treatment was unable to decrease the elevated levels of activated caspase-3, (Figure 5.5.2B).

Figure 5.5.1 Cholinergic mediated tau hyperphosphorylation is regulated by GSK3 activation. (A) Representative Western-blot and quantification analysis of pAKT(S473) and pAKT(Thr308) levels in the hippocampus of VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice. (n=4, data are mean ± SEM. *P<0.05 (B) Representative Western blot and quantification analysis of phospho-GSK3α and β in the hippocampus of aged (11-14 month old) controls (left) and VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ (right) mice. Levels of pGSK3α and β to the respective GSK3 (n= 5 and 3, data are mean ± SEM. **P<0.01, ***P<0.001). (C) Implantation of osmotic pumps and delivery of AR-A014418 to aged VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ mice. (D) Representative Western blot and quantification analysis of phospho-GSK3α and β in the hippocampus of aged VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ mice treated with AR-A014418 or saline. (E) Western blot analysis aged VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ treated with AR-A014418 or saline for Tau hyper-phosphorylation at T231, MCI immunopositive tau and total Tau protein expression. (F) Representative immunolabelling of reduced T231 Tau in the hippocampus of aged VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ mice (Scale bar, 100µm). (G) Western blot analysis for PSD-95 protein levels in the hippocampus of VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice treated with AR-A014418 or saline. (n= 4 saline treated, n=5 AR-A014418 treated, data are mean ± SEM. *P<0.05 ***P<0.001).

Figure 5.5.1 Cholinergic mediated tau hyperphosphorylation is regulated by GSK3 activation.

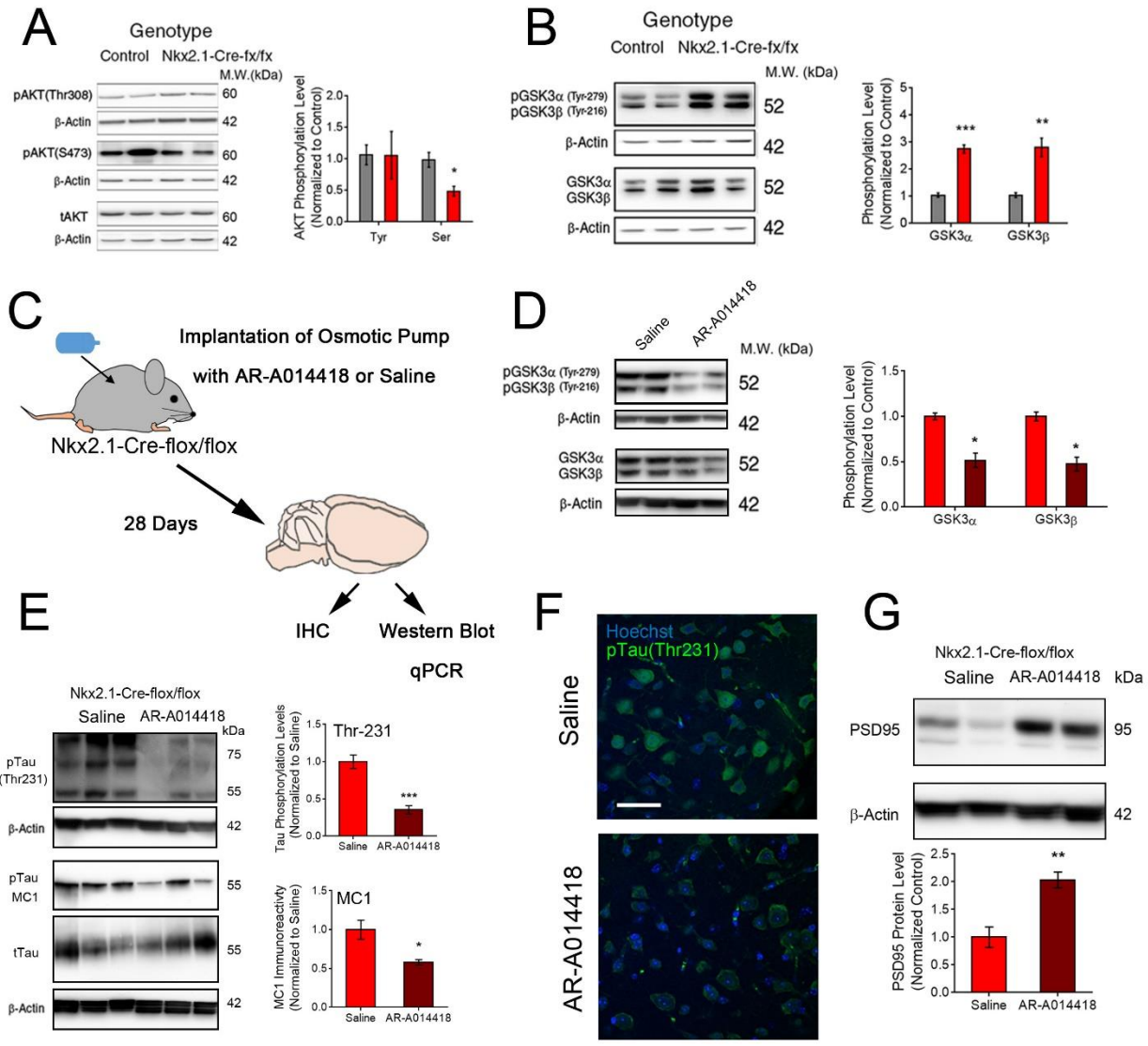
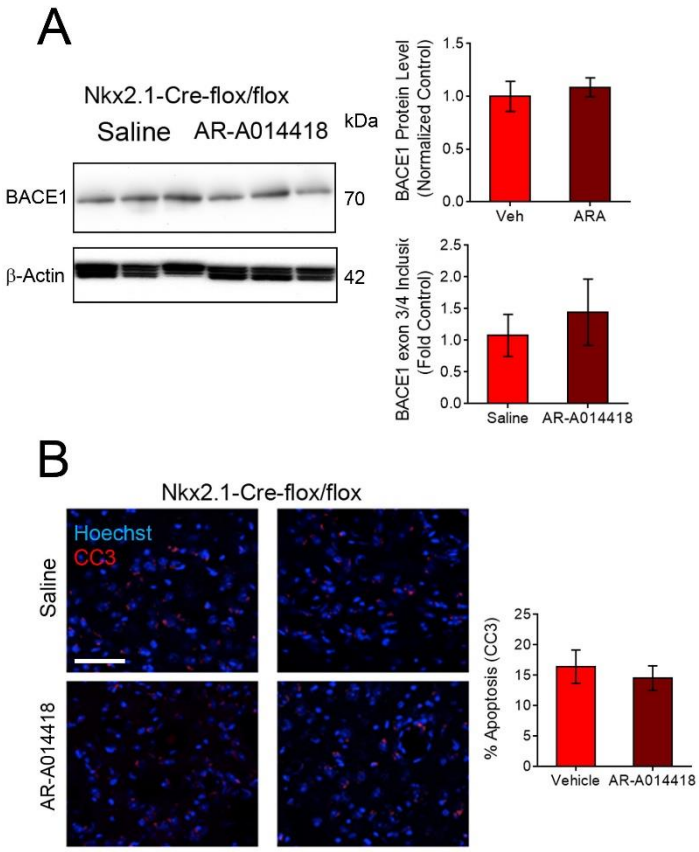


Figure 5.5.2 Cholinergic mediated age dependent pathology is partially mediated by GSK3 hyper-phosphorylation. (A) Representative Western blot and quantification analysis of BACE1 protein levels, along with qPCR analysis of alternative splicing of exon 3/4 of the BACE1 gene in hippocampus of $VAChT^{NKx2.1\text{-}Cre\text{-}flox/flox}$ treated with AR-A014418 or saline. (B) Quantification of activated caspase-3 immunolabelling in the hippocampus of $VAChT^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice treated with AR-A014418 or saline (Scale bar, 100µm) (n= 4 saline treated, n=5 AR-A014418 treated, data are mean ± SEM. *P<0.05 **P<0.01).

Figure 5.5.2 Cholinergic mediated age dependent pathology is partially mediated by GSK3 hyper-phosphorylation.



5.4.6 Cholinergic dysfunction in human AD brains

Whether cholinergic genes are expressed in lower levels in human AD brain compared to cognitively alert controls and may contribute to phenotypes detected herein is not fully understood. To examine that, we extracted total RNA from a cohort of 24 adult human temporal gyrus samples collected at the Netherland Brain Bank (sample information in Table 5.61), including 8 sporadic AD patients and 16 from age-matched controls. We then profiled AD-related transcript differences by adopting the particularly deep SQUARETM RNA library construction approach (Hebenstreit and Teichmann, 2011). Of those, 10,885 genes that were expressed showed a significant change. Next, we quantified the levels of those transcripts composing the expanded family of cholinergic regulator genes (Soreq, 2015). Detected cholinergic transcripts showed significantly lower expression levels than other protein-coding genes in the temporal gyrus of AD patients compared to aged-matched controls (Figure 5.6A).

Brain samples from a distinct cohort of AD patients (Ostapchenko et al., 2013) supported the RNA-Seq analysis results by showing a significant VAChT loss in AD brains compared to age and sex-matched controls (Figure 5.6B), in agreement with previous observations (Chen et al., 2011; Efange et al., 1997). Furthermore, the cohort of AD brains exhibited 50% decrease in hnRNPA2/B1 protein levels compared to age/gender-matched controls (Figure 5.6C), confirming previous results obtained with a distinct AD cohort (Berson et al., 2012). Additionally, we found a significant positive correlation between VAChT and hnRNPA2/B1 protein levels (Figure 5.6D). AD brain samples also showed drastic increases in tau-Thr231 phosphorylation (Figure 5.6E), which was inversely proportional to the levels of VAChT (Figure 5.6F). Our findings using cholinergic-deficient mice support an intricate timeline whereby cholinergic dysfunction per se precedes and may have strong influence in pathological changes observed in AD.

Figure 5.6 Cholinergic Failure in the human AD Brain associates with loss of hnRNPA2/B1 and hyperphosporylation of tau. (A) Cholinergic Genes are down-regulated in the AD temporal gyrus. Shown are cumulative distribution functions (CDFs) for the global change in the expanded family of cholinergic genes (as listed in Soreq, 2015) compared to global expression patterns between AD and control brain tissues (n= 8, Kolmogorov Smirnov p=0.03, red and blue lines, correspondingly). (B) Western blot analysis of VAChT protein levels in AD brains. (C) Western blot analysis of hnRNPA2/B1 protein levels in AD brains. (D) Correlation between hnRNPA2/B1 protein levels and VAChT protein levels in AD brains. (E) Western blot analysis of Tau-Thr231 phosphorylation levels and (F) correlation between VAChT protein levels and Tau Thr-231 phosphorylation levels. (n= 6, data are mean ± SEM. *P<0.05 **P<0.01).

Figure 5.6 Cholinergic Failure in the human AD Brain associates with loss of hnRNPA2/B1 and hyperphosporylation of tau.

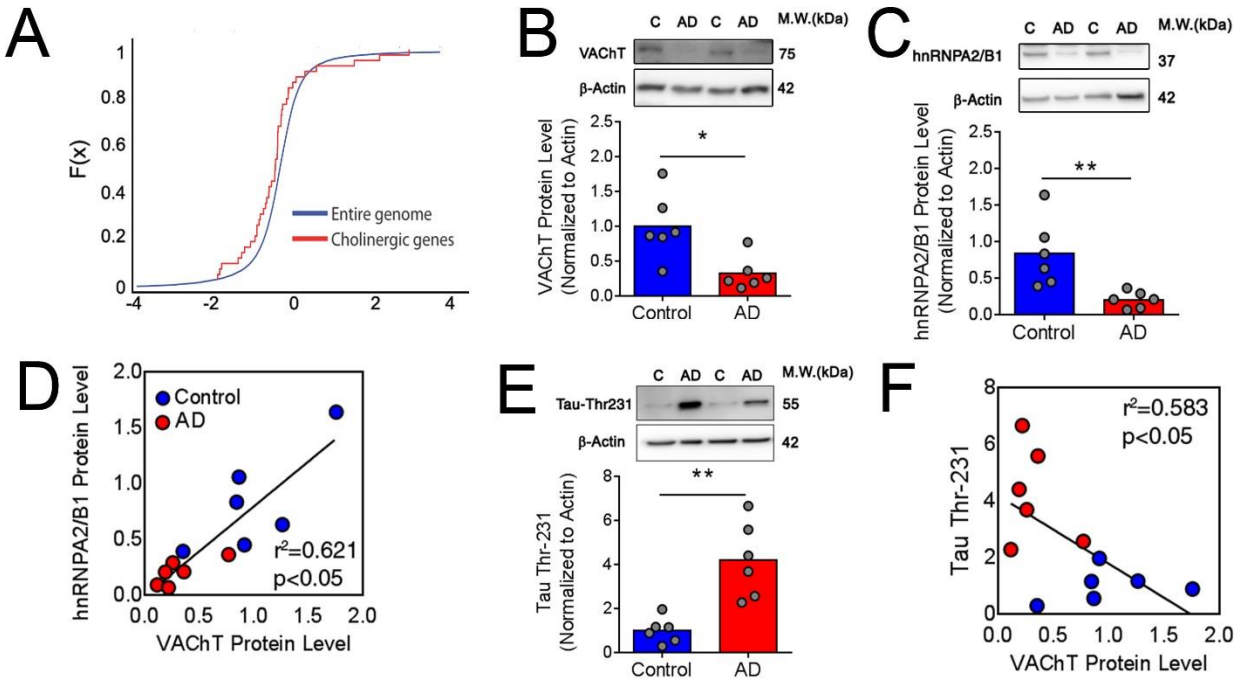


Table 5.6.1 Data for cohort of human brain samples. Samples collected from the Netherland Brain Bank, from which total RNA was extracted and sequenced, n=24.

Table 5.6.1 Data for cohort of human brain samples.

NBB id	sex	age	Post mortem delay	ph	weight	region
2008-047	M	77	06:35	6.10	1250	superior temporalis gyrus
2000-066	M	80	04:20	7.08	1160	inferior temporalis gyrus
2007-025	M	82	05:15	6.34	1182	superior temporalis gyrus
2007-052	M	82	04:15	6.41	1205	medial temporalis gyrus
2009-040	M	83	06:10	5.91	1102	superior temporalis gyrus
2008-029	M	84	08:05	5.95	1195	superior temporalis gyrus
2001-044	M	85	04:25	6.20	1383	superior temporalis gyrus
2001-063	M	85	04:45	6.38	1215	superior temporalis gyrus
2010-016	M	86	06:15	?	1211	superior temporalis gyrus
2009-107	M	88	04:40	6.22	1054	superior temporalis gyrus
2005-010	M	93	04:30	6.46	1040	superior temporalis gyrus
2002-087	M	71	07:40	6.20	1150	superior temporalis gyrus
2001-016	M	77	08:25	7.19	1118	superior temporalis gyrus
2000-015	M	78	05:35	6.63	1417	inferior temporalis gyrus
2005-044	M	80	07:15	5.80	1331	superior temporalis gyrus
2001-021	M	82	07:40	6.07	1318	inferior temporalis gyrus
2009-005	M	82	05:10	6.75	1087	superior temporalis gyrus
2001-086	M	88	07:00	6.84	1368	superior temporalis gyrus
2003-035	M	96	06:30	6.65	1300	superior temporalis gyrus
2005-019	M	74	05:00	6.70	1115	superior temporalis gyrus
2003-084	M	82	10:00	6.53	1488	superior temporalis gyrus
2009-039	M	82	12:55	6.21	1406	superior temporalis gyrus
2005-073	M	87	06:05	6.96	1468	superior temporalis gyrus
2009-075	M	88	07:00	6.76	1230	superior temporalis gyrus

Table 5.6.2 Number of detected genes per patient. Number of detected genes (RPKM>1) per patient, per SQUARE field. Rows are patients and columns are SQUARE fields.

Table 5.6.2 Number of detected genes per patient.

		1	2	3	4	5	6	7	8	9	10	11	12
AD	1	6152	6729	6154	7224	9942	6284	4273	5176	6504	7879	5815	7500
	2	6961	6995	6251	7516	9611	6478	4549	5294	6698	7778	6204	7747
	3	6945	7107	6627	7388	9746	6221	4529	5037	6789	7983	6145	6059
	4	6811	6704	6530	7555	9664	6193	2877	4868	6250	7976	6246	8079
	5	6123	6417	5848	7628	9529	5953	4637	4639	6140	7481	5814	7654
	6	6956	7093	6308	7469	9927	6730	4542	4951	6091	7465	5699	6550
	7	6310	6217	5749	7176	9410	5910	4393	4695	6247	7203	5531	7122
	8	6770	6999	6436	7352	9675	6730	4451	5233	6761	7500	6114	7589
Con	9	6374	6456	5957	6785	9250	6039	3889	4726	6506	7182	5307	6633
	10	6820	6919	6083	6883	9270	5950	4367	4861	6763	7729	5266	6545
	11	5771	6029	5191	6705	9445	5654	4197	4565	6188	6804	5649	6986
	12	5660	7181	6486	7208	10160	7867	5148	5422	6939	8082	6243	6555
	13	6332	6962	6220	6468	9430	6576	3876	5126	6959	7805	5361	6657
	14	6103	6486	5507	7139	9583	5781	4467	4604	6150	7328	5632	7412
	15	6380	6414	5592	6486	9377	5557	3940	4277	6225	7869	5571	7090
	16	6511	6673	6217	7495	9710	6147	4238	4947	6788	8011	5732	7296
	17	6265	6450	5903	7247	9142	5907	4403	4718	6022	7212	5424	7478
	18	7153	7552	6945	7596	9822	6549	4382	5424	7282	8388	5926	7285
	19	6294	5943	5668	7300	9259	5648	4255	4416	6108	7468	5690	7321
	20	6268	6583	5753	7391	9732	6125	4635	4815	5896	7847	5733	7395
	21	7674	7822	6969	8201	10588	7826	5027	5787	7348	8095	6059	7434
	22	7274	7185	6793	7824	9945	6982	4755	5377	7258	8310	6185	7737
	23	7149	7073	6654	7206	9895	6774	4890	5274	6915	8485	5796	8190
	24	5676	6113	5303	6938	9863	6393	4630	4835	6651	7721	5964	6474

5.5 Discussion

We employed transcriptome and biochemical assays on cholinergic-deficient mouse brain samples to explore the impact of long-lasting forebrain cholinergic dysfunction. Whole-genome RNA sequencing demonstrated that cholinergic deficiency modifies expression levels and isoform abundance of several key transcripts related to Alzheimer's disease in the hippocampus of VAChT-deficient mice. Cholinergic-mediated abnormal BACE1 mRNA splicing in VAChT-mutant mice increased BACE1 protein levels and APP processing. Accordingly, cholinergic deficiency caused a 10-fold increase in soluble mouse Aβ peptides, age-dependent hippocampal tauopathy, synaptic abnormalities, neuronal inflammation, neuronal vulnerability and cognitive decline. We also showed that GSK3 activation is critical for cholinergic modulation of tau hyperphosphorylation and synaptic vulnerability. Furthermore, we confirmed that human AD brains present cholinergic dysfunction and showed that it correlates to changes in the levels of hnRNP A2/B1 and hyperphosphorylated tau. Our findings indicate that cholinergic impairments confer widespread hippocampal damage and malfunction. Furthermore, our data support a causal role for cholinergic signalling as a surveillance mechanism controlling hippocampal transcript levels, maintenance of cognitive function and neuronal viability in mice.

Our RNA sequencing analysis revealed a group of differentially expressed transcripts related to spliceosome regulation in the hippocampus of VAChTNkx2.1-Cre-flox/flox mice, suggesting that the splicing machinery in these mutants could be altered. In fact, a significant number of alternative splicing event abnormalities were observed in the hippocampus of VAChT-deficient mice. These results are consistent with previously reported global changes of alternative splicing in the AD brain (Bai et al., 2013; Berson et al., 2012; Tollervey et al., 2011). Importantly, spliceosome signalling pathway changes can have broad implications for gene regulation [reviewed in (Shin and Manley, 2004)].

Alternative splicing in the nervous system is particularly widespread and is essential for multiple aspects of neuronal function (Raj and Blencowe, 2015). However, the signal-

transduction pathways that regulate splicing are not well known (Shin and Manley, 2004). Our study adds a role for cholinergic signalling in the maintenance of balanced alternative splicing. At least part of the cholinergic-control of alternative splicing seems to involve hnRNPA2/B1. We have shown that cholinergic deficiency in the cortex (Berson et al., 2012; Kolisnyk et al., 2013a) and hippocampus (Figure 2B) leads to decreased expression of the hnRNPA2/B1 protein. Related work demonstrated that hnRNPA2/B1 is a cholinergic regulated splicing factor (Kolisnyk et al submitted). Importantly, knockdown of hnRNPA2/B1 in cultured hippocampal neurons shifted splicing of BACE1 mRNA towards increased expression of mRNA species coding for the BACE1-501 protein isoform as observed in the hippocampus of VAChT-deficient mice. This splicing change led to increased expression of the BACE1 protein that was accompanied by a pattern of APP processing similar to that observed in commonly used mouse models of AD. The alternative splicing event in BACE1 observed in VAChT-mutant mice is regulated my M1 muscarinic receptors (Kolisnyk et al., submitted).

Cholinergic tone has been thought to regulate APP processing through muscarinic receptors (Davis et al., 2010; Nitsch et al., 1992). Specifically, M1 signalling has been shown to regulate the stability of the BACE1 protein (Davis et al., 2010). BACE1-501 is a more stable and active form of the protein (Mowrer and Wolfe, 2008). Remarkably, BACE1 expression is increased in late-onset AD (Hebert et al., 2008). Our data suggest potential mechanisms by which cholinergic regulation can affect BACE1 expression and AD pathology. Interestingly, our data suggest changes in alternative splicing occurs post-transcriptionally and independent of GSK3 signalling. Thus, cholinergic deficiency may affect BACE1 expression differently than previously described in AD, in which GSK3 can regulate BACE1 transcription by increasing promoter activity (Ly et al., 2013). Our findings promote upstream cholinergic mechanisms as a target for diminishing aberrant APP processing in AD.

In addition to increased levels of soluble Aβ, VAChTNkx2.1-Cre-flox/flox mice also show tau hyper-phosphorylation, which destabilizes microtubules and significantly disrupts axonal transport. Tau hyper-phosphorylation may also contribute to increased vulnerability leading to neuronal death (Billingsley and Kincaid, 1997). In fact, VAChT-

deficient mice show age-dependent increases in hippocampal argyrophilic staining and neuronal death.

Oligomeric protein aggregation has been linked to toxicity and to neurodegenerative disorders, including AD (Maeda et al., 2006). The formation of NFTs alone is insufficient for neurodegeneration, yet oligomeric tau may contribute to neurodegeneration and synaptic loss in AD (Berger et al., 2007; de Calignon et al., 2012). These observed changes in tau in VAChT-deficient mice (i.e. increased oligomer formation), the associated age-dependent increase in immunoreactivity of activated caspase-3, and ultimately neuronal loss all support a relationship between cholinergic failure and AD-like pathology in mice.

Tau hyper-phosphorylation can occur due to the increased activity of GSK3, which subsequently leads to an array of impairments, including disruption of LTP (Hooper et al., 2007) and cell death in vitro (Zheng et al., 2002). GSK3 over-activation is an important hallmark in AD (Hooper et al., 2008). Thus, the GSK3 overactivation observed in VAChT-mutant mice represents a potential mechanism by which reduced cholinergic activity may have multiple influences in AD pathology in target cells. We tested this hypothesis by pharmacological inhibition of GSK3 in aged VAChT-deficient mice. GSK3 inhibition was able to decrease tau hyperphosphorylation. Also, GSK3 inhibition partially restored PSD95 protein levels, but did not decrease caspase-3 activation. These findings demonstrate that cholinergic-induced changes in tau and in amyloid processing are potentially independent of each other and suggest that cholinergic dysfunction is contributing to the pathological outcomes in these animals by altering multiple pathways. The pharmacological inhibition of GSK3 was tested in mice in which certain pathology was already present (11-12 month-old mice). Hence, further experiments should test longer treatments with the compound or genetic ablation of GSK3 genes in VAChT-deficient mice to comprehensively dissect the contribution of overactive GSK3 in other phenotypes.

Aged VAChT-deficient mice showed a decrease in the number of hippocampal neurons, a deficiency that was not observed in young mutants, suggesting that cholinergic tone may play a role in guarding hippocampal neuronal health. Additionally, aged VAChT-

deficient mice showed increased neuroinflammation and reduced number of synapses; which are pathologies observed in AD brains (DeKosky and Scheff, 1990; Rogers and Shen, 2000; Smale et al., 1995). Mice with excess acetylcholinesterase, which present decreased cholinergic function, also show neuroinflammation (Shaked et al., 2009). Furthermore, similar to our observation, mice lacking the β2 nicotinic receptor subunit show age-dependent loss of hippocampal neurons (Zoli et al., 1999). Of note, hippocampal neuronal loss is a critical feature in AD, which is not observed in mouse models overexpressing APP and or presenilin 1 with human AD mutations (Stein and Johnson, 2002). Hence, long-term cholinergic deficiency may model this aspect of AD in a better way. Potential mechanisms involved in cholinergic dysfunction induced pathology are shown on Figure 5.7.

In line with an age-dependent loss of hippocampal neurons, we found that aged VAChT-deficient mice showed significant impairments in their learning strategy in the MWM task. Poor performance and acquisition on the MWM task has been associated with loss of neurons in the hippocampus (Olsen et al., 1994). This suggests that neuronal loss in the hippocampus has functional consequences in mice as well.

In AD brains, we found evidence of cholinergic decline and showed a striking relationship between VAChT levels and tau hyper-phosphorylation. Together with the mouse data, these observations support the notion that deficient cholinergic signalling in AD may correlate to key pathological events, including Tau hyper-phosphorylation.

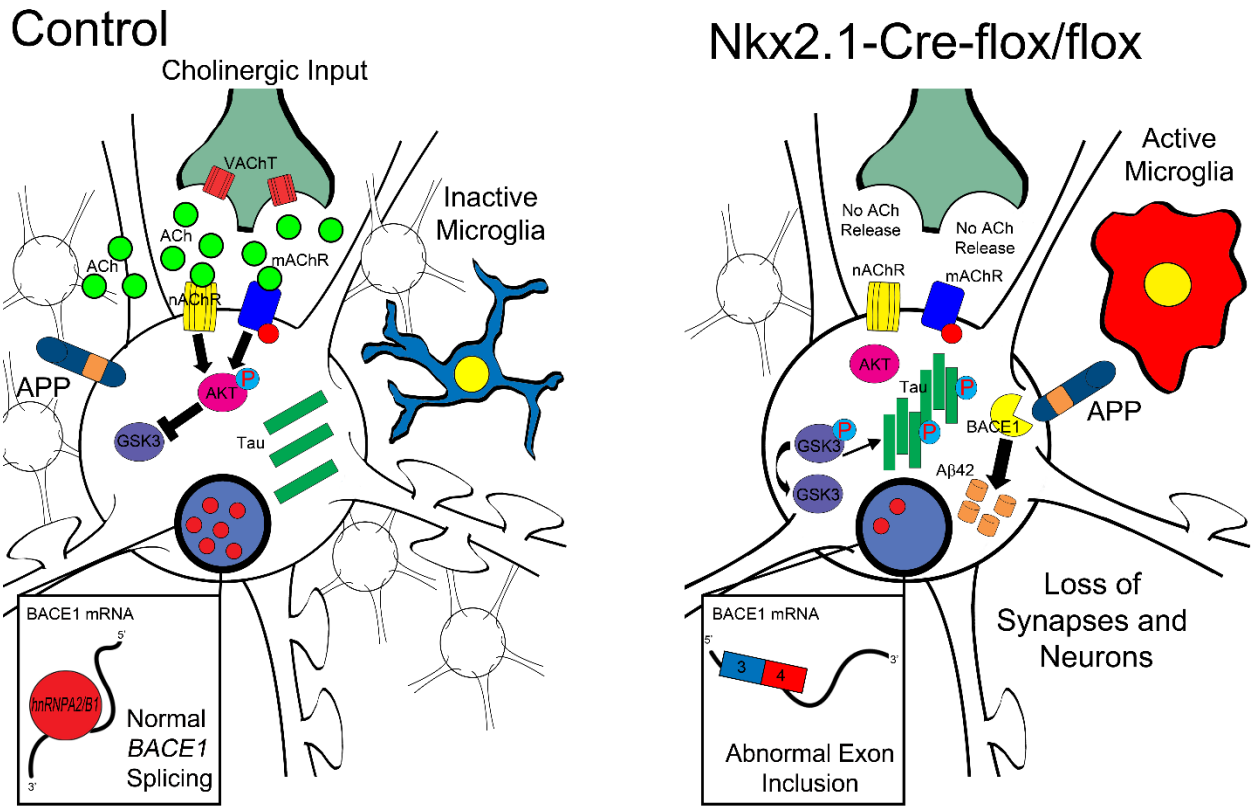
Our data reveal that cholinergic deficiency can affect a number of transcriptional processes, disturb splicing of key genes and interfere with protein networks that normally protect neurons. Interestingly, recent work revealed that basal forebrain cholinergic neurons present intraneuronal Aβ accumulation even in young adults, which may contribute to their selective vulnerability in AD (Baker-Nigh et al., 2015). Cholinergic neurons are thought to be highly dependent on the presence of trophic factors for their optimal function and survival (Boskovic et al., 2014; Naumann et al., 2002), which may also contribute to their demise. Regardless of the mechanisms for increased cholinergic vulnerability in AD, it seems that cholinergic dysfunction persisting

for a long period is highly related to hippocampal pathology and amyloid accumulation (Teipel et al., 2014).

In short, our results suggest that long-term cholinergic failure per se, which we model by disrupting synaptic cholinergic function, can trigger AD-like pathology in mice. More importantly, we find that long-term cholinergic deficiency leads to age-dependent cognitive decline that is related to neuronal death, a key feature of late-onset AD that is not modeled in mice overexpressing human genes with AD-related mutations. Our experiments provide a mechanism to explain how decreased cholinergic tone, for example due to long-term use of anti-cholinergic drugs, could lead to increased risk of dementia (Gray et al., 2015), which may depend on global changes of RNA metabolism, including alternative splicing and gene expression. It remains to be determined if rescuing cholinergic function prior to development of AD could have an impact in the risk of dementia or AD-related pathology. However, it is noteworthy that recent observations in potential prodromal AD-affected individuals indicate that cholinesterase inhibition decreases the rate of hippocampal atrophy by 45% during one-year treatment (Dubois et al., 2015). Our data points towards cholinergic signalling being a global mediator of several distinct processes that when dysfunctional lead to pathology. Developing effective strategies to reverse the cholinergic deficits in the AD brain may therefore prove to be more fruitful then specific therapies based on reversing the individual processes it regulates.

Figure 5.7. Summary of findings. (A) In control animals, cholinergic input from the medial septum regulates target neurons in the hippocampus, through nicotinic and muscarinic acetylcholine receptors. Long-term cholinergic signaling maintains transcriptome integrity likely by a combination of muscarinic and nicotinic activation. These maintain balance of signaling pathways that regulate AD-like pathology. (B) Conversely, in cholinergic deficient mice, which models long-term cholinergic dysfunction, lack of signaling by muscarinic and nicotinic receptors affects differential expression of spliceosome-related genes and reductions in hnRNPA2/B1. BACE1 mRNA is abnormally spliced leading to an increase of BACE1 expression. As a consequence, APP processing is altered, yielding accumulation of soluble Aβ peptides. Furthermore, abnormal gene expression influences AKT-GSK3 modulatory genes with consequences for AKT and GSK3 phosphorylation. These changes contribute to increases in pathological tau phosphorylation and misfolding, neuroinflammation, synaptic loss, hippocampal neuronal death and ultimately leading to cognitive decline in these animals.

Figure 5.7. Summary of findings.



5.6 Acknowledgements

The authors declare no competing financial interests. They are grateful to Dr. David E. Greenberg, Hebrew University of Jerusalem, for fruitful discussions; to Dr. Alexander Seitz and Dr. Torsten Reda, Lexogen, Vienna, and Mr. Alessandro Guffanti, Genomnia, Milan, for technical and analytic support; the Netherland Brain bank for tissues and data; Jue Fan, Sandra Raulic and Matthew Cowan for animal care and technical support; to Dr. R. Jane Rylett (University of Western Ontario, Canada) and Dr. Peter Davies (Albert Einstein College of Medicine of Yeshiva University, USA) for providing antibodies; and to the Center for Applied Genomics, SickKids, Toronto for RNA-Seq experiments. This work was supported by the Canadian Institute of Health Research (MOP 93651, MOP 136930, MOP 126000 and MOP 89919), NSERC (402524-2013), Brain Canada, Canadian Foundation for Innovation, and Ontario research fund (M.A.M.P. And V.F.P.). H.S acknowledges support by the European Research Council (Advanced Award 321501), and the Legacy Heritage Science Initiative (LHSI) of the Israel Science Foundation (Grant No. 378/11). S.B. is an incumbent of the TEVA National Network of Excellence in Neuroscience – NNE fellowship. B.K. is a recipient of the Annie Darkens Research Fund Award from the Alzheimer's Society of Canada fellowship and M.A.A-O received fellowship support from Kuwait University. JSS received a fellowship from the Science without borders program (Brazil). L.S. was funded by a Marie Curie Actions Intra European Fellowship, the European Commission (call FP7-PEOPLE-2013-IEF, project: PRANA).

5.7 References

Al-Onaizi, M.A., Parfitt, G.M., Kolisnyk, B., Law, C.S., Guzman, M.S., Barros, D.M., Leung, L.S., Prado, M.A., and Prado, V.F. (2016). Regulation of Cognitive Processing by Hippocampal Cholinergic Tone. Cerebral cortex.

Anders, S., and Huber, W. (2010). Differential expression analysis for sequence count data. Genome biology 11, R106.

Bai, B., Hales, C.M., Chen, P.C., Gozal, Y., Dammer, E.B., Fritz, J.J., Wang, X., Xia, Q., Duong, D.M., Street, C., et al. (2013). U1 small nuclear ribonucleoprotein complex and RNA splicing alterations in Alzheimer's disease. Proceedings of the National Academy of Sciences of the United States of America 110, 16562-16567.

Baker-Nigh, A., Vahedi, S., Davis, E.G., Weintraub, S., Bigio, E.H., Klein, W.L., and Geula, C. (2015). Neuronal amyloid-beta accumulation within cholinergic basal forebrain in ageing and Alzheimer's disease. Brain : a journal of neurology 138, 1722-1737.

Bartus, R.T., Dean, R.L., 3rd, Beer, B., and Lippa, A.S. (1982). The cholinergic hypothesis of geriatric memory dysfunction. Science 217, 408-414.

Beauquis, J., Vinuesa, A., Pomilio, C., Pavia, P., Galvan, V., and Saravia, F. (2014). Neuronal and glial alterations, increased anxiety, and cognitive impairment before hippocampal amyloid deposition in PDAPP mice, model of Alzheimer's disease. Hippocampus 24, 257-269.

Bekenstein, U., and Soreq, H. (2013). Heterogeneous nuclear ribonucleoprotein A1 in health and neurodegenerative disease: from structural insights to post-transcriptional regulatory roles. Molecular and cellular neurosciences 56, 436-446.

Berger, Z., Roder, H., Hanna, A., Carlson, A., Rangachari, V., Yue, M., Wszolek, Z., Ashe, K., Knight, J., Dickson, D., et al. (2007). Accumulation of pathological tau species and memory loss in a conditional model of tauopathy. The Journal of neuroscience : the official journal of the Society for Neuroscience 27, 3650-3662.

Berson, A., Barbash, S., Shaltiel, G., Goll, Y., Hanin, G., Greenberg, D.S., Ketzef, M., Becker, A.J., Friedman, A., and Soreq, H. (2012). Cholinergic-associated loss of hnRNP-A/B in Alzheimer's disease impairs cortical splicing and cognitive function in mice. EMBO Mol Med 4, 730-742.

Billingsley, M.L., and Kincaid, R.L. (1997). Regulated phosphorylation and dephosphorylation of tau protein: effects on microtubule interaction, intracellular trafficking and neurodegeneration. The Biochemical journal 323 (Pt 3), 577-591.

Bindea, G., Mlecnik, B., Hackl, H., Charoentong, P., Tosolini, M., Kirilovsky, A., Fridman, W.H., Pages, F., Trajanoski, Z., and Galon, J. (2009). ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics 25, 1091-1093.

Boskovic, Z., Alfonsi, F., Rumballe, B.A., Fonseka, S., Windels, F., and Coulson, E.J. (2014). The role of p75NTR in cholinergic basal forebrain structure and function. The Journal of neuroscience : the official journal of the Society for Neuroscience 34, 13033-13038.

Carter, Y.M., Kunnimalaiyaan, S., Chen, H., Gamblin, T.C., and Kunnimalaiyaan, M. (2014). Specific glycogen synthase kinase-3 inhibition reduces neuroendocrine markers and suppresses neuroblastoma cell growth. Cancer biology & therapy 15, 510-515.

Chai, X., Wu, S., Murray, T.K., Kinley, R., Cella, C.V., Sims, H., Buckner, N., Hanmer, J., Davies, P., O'Neill, M.J., et al. (2011). Passive immunization with anti-Tau antibodies in two transgenic models: reduction of Tau pathology and delay of disease progression. The Journal of biological chemistry 286, 34457-34467.

Chen, K.H., Reese, E.A., Kim, H.W., Rapoport, S.I., and Rao, J.S. (2011). Disturbed neurotransmitter transporter expression in Alzheimer's disease brain. Journal of Alzheimer's disease : JAD 26, 755-766.

Davis, A.A., Fritz, J.J., Wess, J., Lah, J.J., and Levey, A.I. (2010). Deletion of M1 muscarinic acetylcholine receptors increases amyloid pathology in vitro and in vivo. The Journal of neuroscience : the official journal of the Society for Neuroscience 30, 4190-4196.

de Calignon, A., Polydoro, M., Suarez-Calvet, M., William, C., Adamowicz, D.H., Kopeikina, K.J., Pitstick, R., Sahara, N., Ashe, K.H., Carlson, G.A., et al. (2012). Propagation of tau pathology in a model of early Alzheimer's disease. Neuron 73, 685-697.

de Castro, B.M., De Jaeger, X., Martins-Silva, C., Lima, R.D., Amaral, E., Menezes, C., Lima, P., Neves, C.M., Pires, R.G., Gould, T.W., et al. (2009). The vesicular

acetylcholine transporter is required for neuromuscular development and function. Mol Cell Biol 29, 5238-5250.

DeKosky, S.T., and Scheff, S.W. (1990). Synapse loss in frontal cortex biopsies in Alzheimer's disease: correlation with cognitive severity. Annals of neurology 27, 457-464.

DeOlmos, J.S., and Ingram, W.R. (1971). An improved cupric-silver method for impregnation of axonal and terminal degeneration. Brain research 33, 523-529.

Dewachter, I., Reverse, D., Caluwaerts, N., Ris, L., Kuiperi, C., Van den Haute, C., Spittaels, K., Umans, L., Serneels, L., Thiry, E., et al. (2002). Neuronal deficiency of presenilin 1 inhibits amyloid plaque formation and corrects hippocampal long-term potentiation but not a cognitive defect of amyloid precursor protein [V717I] transgenic mice. The Journal of neuroscience : the official journal of the Society for Neuroscience 22, 3445-3453.

Dubois, B., Chupin, M., Hampel, H., Lista, S., Cavedo, E., Croisile, B., Louis Tisserand, G., Touchon, J., Bonafe, A., Ousset, P.J., et al. (2015). Donepezil decreases annual rate of hippocampal atrophy in suspected prodromal Alzheimer's disease. Alzheimer's & dementia : the journal of the Alzheimer's Association 11, 1041-1049.

Eden, E., Navon, R., Steinfeld, I., Lipson, D., and Yakhini, Z. (2009). GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. BMC bioinformatics 10, 48.

Efange, S.M., Garland, E.M., Staley, J.K., Khare, A.B., and Mash, D.C. (1997). Vesicular acetylcholine transporter density and Alzheimer's disease. Neurobiology of aging 18, 407-413.

Endo, H., Nito, C., Kamada, H., Nishi, T., and Chan, P.H. (2006). Activation of the Akt/GSK3beta signaling pathway mediates survival of vulnerable hippocampal neurons after transient global cerebral ischemia in rats. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 26, 1479-1489.

Garthe, A., Behr, J., and Kempermann, G. (2009). Adult-generated hippocampal neurons allow the flexible use of spatially precise learning strategies. PloS one 4, e5464.

Gary, D.S., and Mattson, M.P. (2001). Integrin signaling via the PI3-kinase-Akt pathway increases neuronal resistance to glutamate-induced apoptosis. Journal of neurochemistry 76, 1485-1496.

Goedert, M., Jakes, R., Crowther, R.A., Cohen, P., Vanmechelen, E., Vandermeeren, M., and Cras, P. (1994). Epitope mapping of monoclonal antibodies to the paired helical filaments of Alzheimer's disease: identification of phosphorylation sites in tau protein. The Biochemical journal 301 (Pt 3), 871-877.

Gray, S.L., Anderson, M.L., Dublin, S., Hanlon, J.T., Hubbard, R., Walker, R., Yu, O., Crane, P.K., and Larson, E.B. (2015). Cumulative use of strong anticholinergics and incident dementia: a prospective cohort study. JAMA internal medicine 175, 401-407.

Grothe, M., Heinsen, H., and Teipel, S. (2013). Longitudinal measures of cholinergic forebrain atrophy in the transition from healthy aging to Alzheimer's disease. Neurobiology of aging 34, 1210-1220.

Grothe, M.J., Ewers, M., Krause, B., Heinsen, H., Teipel, S.J., and Alzheimer's Disease Neuroimaging, I. (2014). Basal forebrain atrophy and cortical amyloid deposition in nondemented elderly subjects. Alzheimer's & dementia : the journal of the Alzheimer's Association 10, S344-353.

Guzman, M.S., De Jaeger, X., Raulic, S., Souza, I.A., Li, A.X., Schmid, S., Menon, R.S., Gainetdinov, R.R., Caron, M.G., Bartha, R., et al. (2011). Elimination of the vesicular acetylcholine transporter in the striatum reveals regulation of behaviour by cholinergic-glutamatergic co-transmission. PLoS Biol 9, e1001194.

Hebenstreit, D., and Teichmann, S.A. (2011). Analysis and simulation of gene expression profiles in pure and mixed cell populations. Physical biology 8, 035013.

Hebert, S.S., Horre, K., Nicolai, L., Papadopoulou, A.S., Mandemakers, W., Silahtaroglu, A.N., Kauppinen, S., Delacourte, A., and De Strooper, B. (2008). Loss of

microRNA cluster miR-29a/b-1 in sporadic Alzheimer's disease correlates with increased BACE1/beta-secretase expression. Proceedings of the National Academy of Sciences of the United States of America 105, 6415-6420.

Hellstrom-Lindahl, E. (2000). Modulation of beta-amyloid precursor protein processing and tau phosphorylation by acetylcholine receptors. European journal of pharmacology 393, 255-263.

Hooper, C., Killick, R., and Lovestone, S. (2008). The GSK3 hypothesis of Alzheimer's disease. Journal of neurochemistry 104, 1433-1439.

Hooper, C., Markevich, V., Plattner, F., Killick, R., Schofield, E., Engel, T., Hernandez, F., Anderton, B., Rosenblum, K., Bliss, T., et al. (2007). Glycogen synthase kinase-3 inhibition is integral to long-term potentiation. The European journal of neuroscience 25, 81-86.

Hur, E.M., and Zhou, F.Q. (2010). GSK3 signalling in neural development. Nature reviews Neuroscience 11, 539-551.

Iqbal, K., Liu, F., Gong, C.X., and Grundke-Iqbal, I. (2010). Tau in Alzheimer disease and related tauopathies. Current Alzheimer research 7, 656-664.

Jankowsky, J.L., Fadale, D.J., Anderson, J., Xu, G.M., Gonzales, V., Jenkins, N.A., Copeland, N.G., Lee, M.K., Younkin, L.H., Wagner, S.L., et al. (2004). Mutant presenilins specifically elevate the levels of the 42 residue beta-amyloid peptide in vivo: evidence for augmentation of a 42-specific gamma secretase. Human molecular genetics 13, 159-170.

Jankowsky, J.L., Younkin, L.H., Gonzales, V., Fadale, D.J., Slunt, H.H., Lester, H.A., Younkin, S.G., and Borchelt, D.R. (2007). Rodent A beta modulates the solubility and distribution of amyloid deposits in transgenic mice. The Journal of biological chemistry 282, 22707-22720.

Klein, W.L. (2006). Synaptic targeting by A beta oligomers (ADDLS) as a basis for memory loss in early Alzheimer's disease. Alzheimer's & dementia : the journal of the Alzheimer's Association 2, 43-55.

Kolisnyk, B., Al-Onaizi, M.A., Hirata, P.H., Guzman, M.S., Nikolova, S., Barbash, S., Soreq, H., Bartha, R., Prado, M.A., and Prado, V.F. (2013a). Forebrain deletion of the vesicular acetylcholine transporter results in deficits in executive function, metabolic, and RNA splicing abnormalities in the prefrontal cortex. The Journal of neuroscience : the official journal of the Society for Neuroscience 33, 14908-14920.

Kolisnyk, B., Guzman, M.S., Raulic, S., Fan, J., Magalhaes, A.C., Feng, G., Gros, R., Prado, V.F., and Prado, M.A. (2013b). ChAT-ChR2-EYFP mice have enhanced motor endurance but show deficits in attention and several additional cognitive domains. The Journal of neuroscience : the official journal of the Society for Neuroscience 33, 10427-10438.

Lim, J., Balastik, M., Lee, T.H., Nakamura, K., Liou, Y.C., Sun, A., Finn, G., Pastorino, L., Lee, V.M., and Lu, K.P. (2008). Pin1 has opposite effects on wild-type and P301L tau stability and tauopathy. The Journal of clinical investigation 118, 1877-1889.

Lima Rde, F., Prado, V.F., Prado, M.A., and Kushmerick, C. (2010). Quantal release of acetylcholine in mice with reduced levels of the vesicular acetylcholine transporter. Journal of neurochemistry 113, 943-951.

Luo, Y., Bolon, B., Kahn, S., Bennett, B.D., Babu-Khan, S., Denis, P., Fan, W., Kha, H., Zhang, J., Gong, Y., et al. (2001). Mice deficient in BACE1, the Alzheimer's beta-secretase, have normal phenotype and abolished beta-amyloid generation. Nature neuroscience 4, 231-232.

Ly, P.T., Wu, Y., Zou, H., Wang, R., Zhou, W., Kinoshita, A., Zhang, M., Yang, Y., Cai, F., Woodgett, J., and Song, W. (2013). Inhibition of GSK3beta-mediated BACE1 expression reduces Alzheimer-associated phenotypes. The Journal of clinical investigation 123, 224-235.

Maeda, S., Sahara, N., Saito, Y., Murayama, S., Ikai, A., and Takashima, A. (2006). Increased levels of granular tau oligomers: an early sign of brain aging and Alzheimer's disease. Neuroscience research 54, 197-201.

Martyn, A.C., De Jaeger, X., Magalhaes, A.C., Kesarwani, R., Goncalves, D.F., Raulic, S., Guzman, M.S., Jackson, M.F., Izquierdo, I., Macdonald, J.F., et al. (2012). Elimination of the vesicular acetylcholine transporter in the forebrain causes hyperactivity and deficits in spatial memory and long-term potentiation. Proceedings of the National Academy of Sciences of the United States of America 109, 17651-17656.

Mowrer, K.R., and Wolfe, M.S. (2008). Promotion of BACE1 mRNA alternative splicing reduces amyloid beta-peptide production. The Journal of biological chemistry 283, 18694-18701.

Naumann, T., Casademunt, E., Hollerbach, E., Hofmann, J., Dechant, G., Frotscher, M., and Barde, Y.A. (2002). Complete deletion of the neurotrophin receptor p75NTR leads to long-lasting increases in the number of basal forebrain cholinergic neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 22, 2409-2418.

Nitsch, R.M., Slack, B.E., Wurtman, R.J., and Growdon, J.H. (1992). Release of Alzheimer amyloid precursor derivatives stimulated by activation of muscarinic acetylcholine receptors. Science 258, 304-307.

Oddo, S., Caccamo, A., Shepherd, J.D., Murphy, M.P., Golde, T.E., Kayed, R., Metherate, R., Mattson, M.P., Akbari, Y., and LaFerla, F.M. (2003). Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. Neuron 39, 409-421.

Olsen, G.M., Scheel-Kruger, J., Moller, A., and Jensen, L.H. (1994). Relation of spatial learning of rats in the Morris water maze task to the number of viable CA1 neurons following four-vessel occlusion. Behavioral neuroscience 108, 681-690.

Ostapchenko, V.G., Beraldo, F.H., Mohammad, A.H., Xie, Y.F., Hirata, P.H., Magalhaes, A.C., Lamour, G., Li, H., Maciejewski, A., Belrose, J.C., et al. (2013). The prion protein ligand, stress-inducible phosphoprotein 1, regulates amyloid-beta oligomer toxicity. The Journal of neuroscience : the official journal of the Society for Neuroscience 33, 16552-16564.

Paz, I., Kosti, I., Ares, M., Jr., Cline, M., and Mandel-Gutfreund, Y. (2014). RBPmap: a web server for mapping binding sites of RNA-binding proteins. Nucleic acids research 42, W361-367.

Petry, F.R., Pelletier, J., Bretteville, A., Morin, F., Calon, F., Hebert, S.S., Whittington, R.A., and Planel, E. (2014). Specificity of anti-tau antibodies when analyzing mice models of Alzheimer's disease: problems and solutions. PloS one 9, e94251.

Prado, V.F., Martins-Silva, C., de Castro, B.M., Lima, R.F., Barros, D.M., Amaral, E., Ramsey, A.J., Sotnikova, T.D., Ramirez, M.R., Kim, H.G., et al. (2006). Mice deficient for the vesicular acetylcholine transporter are myasthenic and have deficits in object and social recognition. Neuron 51, 601-612.

Prado, V.F., Roy, A., Kolisnyk, B., Gros, R., and Prado, M.A. (2013). Regulation of cholinergic activity by the vesicular acetylcholine transporter. The Biochemical journal 450, 265-274.

Raj, B., and Blencowe, B.J. (2015). Alternative Splicing in the Mammalian Nervous System: Recent Insights into Mechanisms and Functional Roles. Neuron 87, 14-27.

Rogers, J., and Shen, Y. (2000). A perspective on inflammation in Alzheimer's disease. Annals of the New York Academy of Sciences 924, 132-135.

Selkoe, D.J. (2000). Toward a comprehensive theory for Alzheimer's disease. Hypothesis: Alzheimer's disease is caused by the cerebral accumulation and cytotoxicity of amyloid beta-protein. Annals of the New York Academy of Sciences 924, 17-25.

Shaked, I., Meerson, A., Wolf, Y., Avni, R., Greenberg, D., Gilboa-Geffen, A., and Soreq, H. (2009). MicroRNA-132 potentiates cholinergic anti-inflammatory signaling by targeting acetylcholinesterase. Immunity 31, 965-973.

Shin, C., and Manley, J.L. (2004). Cell signalling and the control of pre-mRNA splicing. Nature reviews Molecular cell biology 5, 727-738.

Smale, G., Nichols, N.R., Brady, D.R., Finch, C.E., and Horton, W.E., Jr. (1995). Evidence for apoptotic cell death in Alzheimer's disease. Experimental neurology 133, 225-230.

Soreq, H. (2015). Checks and balances on cholinergic signaling in brain and body function. Trends in neurosciences 38, 448-458.

Soreq, L., Guffanti, A., Salomonis, N., Simchovitz, A., Israel, Z., Bergman, H., and Soreq, H. (2014). Long non-coding RNA and alternative splicing modulations in Parkinson's leukocytes identified by RNA sequencing. PLoS Comput Biol 10, e1003517.

Stein, T.D., and Johnson, J.A. (2002). Lack of neurodegeneration in transgenic mice overexpressing mutant amyloid precursor protein is associated with increased levels of transthyretin and the activation of cell survival pathways. The Journal of neuroscience : the official journal of the Society for Neuroscience 22, 7380-7388.

Sun, A., Nguyen, X.V., and Bing, G. (2002). Comparative analysis of an improved thioflavin-s stain, Gallyas silver stain, and immunohistochemistry for neurofibrillary tangle demonstration on the same sections. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society 50, 463-472.

Teich, A.F., Patel, M., and Arancio, O. (2013). A reliable way to detect endogenous murine beta-amyloid. PloS one 8, e55647.

Teipel, S., Heinsen, H., Amaro, E., Jr., Grinberg, L.T., Krause, B., Grothe, M., and Alzheimer's Disease Neuroimaging, I. (2014). Cholinergic basal forebrain atrophy predicts amyloid burden in Alzheimer's disease. Neurobiology of aging 35, 482-491.

Thompson, D.F., and Walker, C.K. (2015). A descriptive and historical review of bibliometrics with applications to medical sciences. Pharmacotherapy 35, 551-559.

Tollervey, J.R., Wang, Z., Hortobagyi, T., Witten, J.T., Zarnack, K., Kayikci, M., Clark, T.A., Schweitzer, A.C., Rot, G., Curk, T., et al. (2011). Analysis of alternative splicing associated with aging and neurodegeneration in the human brain. Genome research 21, 1572-1582.

Vorhees, C.V., and Williams, M.T. (2006). Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nature protocols 1, 848-858.

Weaver, C.L., Espinoza, M., Kress, Y., and Davies, P. (2000). Conformational change as one of the earliest alterations of tau in Alzheimer's disease. Neurobiology of aging 21, 719-727.

Whitehouse, P.J., Price, D.L., Struble, R.G., Clark, A.W., Coyle, J.T., and Delon, M.R. (1982). Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. Science 215, 1237-1239.

Wolozin, B.L., Pruchnicki, A., Dickson, D.W., and Davies, P. (1986). A neuronal antigen in the brains of Alzheimer patients. Science 232, 648-650.

Yadav, A.K., Vashishta, V., Joshi, N., and Taneja, P. (2014). AR-A 014418 Used against GSK3beta Downregulates Expression of hnRNPA1 and SF2/ASF Splicing Factors. Journal of oncology 2014, 695325.

Zheng, W.H., Kar, S., and Quirion, R. (2002). Insulin-like growth factor-1-induced phosphorylation of transcription factor FKHRL1 is mediated by phosphatidylinositol 3-kinase/Akt kinase and role of this pathway in insulin-like growth factor-1-induced survival of cultured hippocampal neurons. Molecular pharmacology 62, 225-233.

Bai, B., Hales, C.M., Chen, P.C., Gozal, Y., Dammer, E.B., Fritz, J.J., Wang, X., Xia, Q., Duong, D.M., Street, C.*, et al.* (2013). U1 small nuclear ribonucleoprotein complex and RNA splicing alterations in Alzheimer's disease. Proceedings of the National Academy of Sciences of the United States of America 110, 16562-16567.

Bai, Y., Markham, K., Chen, F., Weerasekera, R., Watts, J., Horne, P., Wakutani, Y., Bagshaw, R., Mathews, P.M., Fraser, P.E.*, et al.* (2008). The in vivo brain interactome of the amyloid precursor protein. Molecular & cellular proteomics : MCP 7, 15-34.

Bakalash, S., Pham, M., Koronyo, Y., Salumbides, B.C., Kramerov, A., Seidenberg, H., Berel, D., Black, K.L., and Koronyo-Hamaoui, M. (2011). Egr1 expression is induced following glatiramer acetate immunotherapy in rodent models of glaucoma and Alzheimer's disease. Investigative ophthalmology & visual science 52, 9033-9046.

Blume-Jensen, P., Janknecht, R., and Hunter, T. (1998). The kit receptor promotes cell survival via activation of PI 3-kinase and subsequent Akt-mediated phosphorylation of Bad on Ser136. Current biology : CB 8, 779-782.

Broer, L., Ikram, M.A., Schuur, M., DeStefano, A.L., Bis, J.C., Liu, F., Rivadeneira, F., Uitterlinden, A.G., Beiser, A.S., Longstreth, W.T.*, et al.* (2011). Association of HSP70 and its co-chaperones with Alzheimer's disease. Journal of Alzheimer's disease : JAD 25, 93-102.

Brooks, W.M., Lynch, P.J., Ingle, C.C., Hatton, A., Emson, P.C., Faull, R.L., and Starkey, M.P. (2007). Gene expression profiles of metabolic enzyme transcripts in Alzheimer's disease. Brain research 1127, 127-135.

Carter, T.A., Greenhall, J.A., Yoshida, S., Fuchs, S., Helton, R., Swaroop, A., Lockhart, D.J., and Barlow, C. (2005). Mechanisms of aging in senescence-accelerated mice. Genome biology 6, R48.

Clarimon, J., Bertranpetit, J., Boada, M., Tarraga, L., and Comas, D. (2003). HSP70-2 (HSPA1B) is associated with noncognitive symptoms in late-onset Alzheimer's disease. Journal of geriatric psychiatry and neurology 16, 146-150.

Clarimon, J., Djaldetti, R., Lleo, A., Guerreiro, R.J., Molinuevo, J.L., Paisan-Ruiz, C., Gomez-Isla, T., Blesa, R., Singleton, A., and Hardy, J. (2009). Whole genome analysis in a consanguineous family with early onset Alzheimer's disease. Neurobiology of aging 30, 1986-1991.

Clemmensen, C., Aznar, S., Knudsen, G.M., and Klein, A.B. (2012). The microtubule-associated protein 1A (MAP1A) is an early molecular target of soluble Abeta-peptide. Cellular and molecular neurobiology 32, 561-566.

Cong, L., Jia, J., Qin, W., Ren, Y., and Sun, Y. (2014). Genome-wide analysis of DNA methylation in an APP/PS1 mouse model of Alzheimer's disease. Acta neurologica Belgica 114, 195-206.

Cozza, A., Melissari, E., Iacopetti, P., Mariotti, V., Tedde, A., Nacmias, B., Conte, A., Sorbi, S., and Pellegrini, S. (2008). SNPs in neurotrophin system genes and Alzheimer's disease in an Italian population. Journal of Alzheimer's disease : JAD 15, 61-70.

Desplats, P.A., Kass, K.E., Gilmartin, T., Stanwood, G.D., Woodward, E.L., Head, S.R., Sutcliffe, J.G., and Thomas, E.A. (2006). Selective deficits in the expression of striatal-enriched mRNAs in Huntington's disease. Journal of neurochemistry 96, 743-757.

Hoerndli, F.J., Pelech, S., Papassotiropoulos, A., and Gotz, J. (2007). Abeta treatment and P301L tau expression in an Alzheimer's disease tissue culture model act synergistically to promote aberrant cell cycle re-entry. The European journal of neuroscience 26, 60-72.

Kalback, W., Esh, C., Castano, E.M., Rahman, A., Kokjohn, T., Luehrs, D.C., Sue, L., Cisneros, R., Gerber, F., Richardson, C*., et al.* (2004). Atherosclerosis, vascular amyloidosis and brain hypoperfusion in the pathogenesis of sporadic Alzheimer's disease. Neurological research 26, 525-539.

Karlsson, O., Berg, A.L., Lindstrom, A.K., Hanrieder, J., Arnerup, G., Roman, E., Bergquist, J., Lindquist, N.G., Brittebo, E.B., and Andersson, M. (2012). Neonatal exposure to the cyanobacterial toxin BMAA induces changes in protein expression and neurodegeneration in adult hippocampus. Toxicological sciences : an official journal of the Society of Toxicology 130, 391-404.

Leroy, K., Duyckaerts, C., Bovekamp, L., Muller, O., Anderton, B.H., and Brion, J.P. (2001). Increase of adenomatous polyposis coli immunoreactivity is a marker of reactive astrocytes in Alzheimer's disease and in other pathological conditions. Acta neuropathologica 102, 1-10.

Liang, D., Han, G., Feng, X., Sun, J., Duan, Y., and Lei, H. (2012). Concerted perturbation observed in a hub network in Alzheimer's disease. PloS one 7, e40498.

Liu, D.X., Biswas, S.C., and Greene, L.A. (2004). B-myb and C-myb play required roles in neuronal apoptosis evoked by nerve growth factor deprivation and DNA damage. The

Journal of neuroscience : the official journal of the Society for Neuroscience 24, 8720-8725.

Ma, Q.L., Zuo, X., Yang, F., Ubeda, O.J., Gant, D.J., Alaverdyan, M., Kiosea, N.C., Nazari, S., Chen, P.P., Nothias, F.*, et al.* (2014). Loss of MAP function leads to hippocampal synapse loss and deficits in the Morris Water Maze with aging. The Journal of neuroscience : the official journal of the Society for Neuroscience 34, 7124-7136.

McGorum, B.C., Pirie, R.S., Eaton, S.L., Keen, J.A., Cumyn, E.M., Arnott, D.M., Chen, W., Lamont, D.J., Graham, L.C., Llavero Hurtado, M.*, et al.* (2015). Proteomic Profiling of Cranial (Superior) Cervical Ganglia Reveals Beta-Amyloid & Ubiquitin Proteasome System Perturbations in an Equine Multiple System Neuropathy. Molecular & cellular proteomics : MCP.

Meda, S.A., Narayanan, B., Liu, J., Perrone-Bizzozero, N.I., Stevens, M.C., Calhoun, V.D., Glahn, D.C., Shen, L., Risacher, S.L., Saykin, A.J., and Pearlson, G.D. (2012). A large scale multivariate parallel ICA method reveals novel imaging-genetic relationships for Alzheimer's disease in the ADNI cohort. NeuroImage 60, 1608-1621.

Meshorer, E., and Soreq, H. (2002). Pre-mRNA splicing modulations in senescence. Aging cell 1, 10-16.

Mohsen, S., Hadi, B., Kourosh, K., Kioomars, S., Mehdi, B., and Khorshid Hamid Reza, K. (2015). Association Study of IL-4 -590 C/T and DDX39B -22 G/C Polymorphisms with The Risk of Late-Onset Alzheimer's Disease in Iranian Population. Current aging science.

Moore, P., White, J., Christiansen, V., and Grammas, P. (1998). Protein kinase C-zeta activity but not level is decreased in Alzheimer's disease microvessels. Neuroscience letters 254, 29-32.

Orre, M., Kamphuis, W., Osborn, L.M., Jansen, A.H., Kooijman, L., Bossers, K., and Hol, E.M. (2014). Isolation of glia from Alzheimer's mice reveals inflammation and dysfunction. Neurobiology of aging 35, 2746-2760.

Passani, L.A., Bedford, M.T., Faber, P.W., McGinnis, K.M., Sharp, A.H., Gusella, J.F., Vonsattel, J.P., and MacDonald, M.E. (2000). Huntingtin's WW domain partners in Huntington's disease post-mortem brain fulfill genetic criteria for direct involvement in Huntington's disease pathogenesis. Human molecular genetics 9, 2175-2182.

Qian, W., and Liu, F. (2014). Regulation of alternative splicing of tau exon 10. Neuroscience bulletin 30, 367-377.

Raj, T., Ryan, K.J., Replogle, J.M., Chibnik, L.B., Rosenkrantz, L., Tang, A., Rothamel, K., Stranger, B.E., Bennett, D.A., Evans, D.A.*, et al.* (2014). CD33: increased inclusion of exon 2 implicates the Ig V-set domain in Alzheimer's disease susceptibility. Human molecular genetics 23, 2729-2736.

Sabo, S., Lambert, M.P., Kessey, K., Wade, W., Krafft, G., and Klein, W.L. (1995). Interaction of beta-amyloid peptides with integrins in a human nerve cell line. Neuroscience letters 184, 25-28.

Schol-Gelok, S., Janssens, A.C., Tiemeier, H., Liu, F., Lopez-Leon, S., Zorkoltseva, I.V., Axenovich, T.I., van Swieten, J.C., Uitterlinden, A.G., Hofman, A.*, et al.* (2010). A genome-wide screen for depression in two independent Dutch populations. Biological psychiatry 68, 187-196.

Scott, S.A., Mufson, E.J., Weingartner, J.A., Skau, K.A., and Crutcher, K.A. (1995). Nerve growth factor in Alzheimer's disease: increased levels throughout the brain coupled with declines in nucleus basalis. The Journal of neuroscience : the official journal of the Society for Neuroscience 15, 6213-6221.

Seenundun, S., and Robaire, B. (2007). Time-dependent rescue of gene expression by androgens in the mouse proximal caput epididymidis-1 cell line after androgen withdrawal. Endocrinology 148, 173-188.

Sherva, R., Baldwin, C.T., Inzelberg, R., Vardarajan, B., Cupples, L.A., Lunetta, K., Bowirrat, A., Naj, A., Pericak-Vance, M., Friedland, R.P., and Farrer, L.A. (2011). Identification of novel candidate genes for Alzheimer's disease by autozygosity mapping using genome wide SNP data. Journal of Alzheimer's disease : JAD 23, 349-359.

Silver, M., Janousova, E., Hua, X., Thompson, P.M., and Montana, G. (2012). Identification of gene pathways implicated in Alzheimer's disease using longitudinal imaging phenotypes with sparse regression. NeuroImage 63, 1681-1694.

Taguchi, K., Yamagata, H.D., Zhong, W., Kamino, K., Akatsu, H., Hata, R., Yamamoto, T., Kosaka, K., Takeda, M., Kondo, I., and Miki, T. (2005). Identification of hippocampus-related candidate genes for Alzheimer's disease. Annals of neurology 57, 585-588.

Tarkowski, E., Issa, R., Sjogren, M., Wallin, A., Blennow, K., Tarkowski, A., and Kumar, P. (2002). Increased intrathecal levels of the angiogenic factors VEGF and TGF-beta in Alzheimer's disease and vascular dementia. Neurobiology of aging 23, 237-243.

Tollervey, J.R., Wang, Z., Hortobagyi, T., Witten, J.T., Zarnack, K., Kayikci, M., Clark, T.A., Schweitzer, A.C., Rot, G., Curk, T.*, et al.* (2011). Analysis of alternative splicing associated with aging and neurodegeneration in the human brain. Genome research 21, 1572-1582.

Walker, D.G., Terai, K., Matsuo, A., Beach, T.G., McGeer, E.G., and McGeer, P.L. (1998). Immunohistochemical analyses of fibroblast growth factor receptor-1 in the human substantia nigra. Comparison between normal and Parkinson's disease cases. Brain research 794, 181-187.

Zhang, L., Guo, X.Q., Chu, J.F., Zhang, X., Yan, Z.R., and Li, Y.Z. (2015). Potential hippocampal genes and pathways involved in Alzheimer's disease: a bioinformatic analysis. Genetics and molecular research : GMR 14, 7218-7232.

Chapter 6

Cholinergic Regulation of hnRNPA2/B1 Translation by M1 Muscarinic Receptors

6.1 Chapter Summary

Cholinergic vulnerability, characterized by loss of acetylcholine (ACh), is one of the hallmarks of Alzheimer's disease (AD). Recent work has suggested that decreased ACh activity in AD may contribute to pathological changes through global alterations in alternative splicing. This occurs, at least partially, via the regulation of the expression of a critical protein family in RNA processing, hnRNP A/B proteins. These proteins regulate several steps of RNA metabolism, including alternative splicing, RNA trafficking, miRNA export and gene expression, providing multilevel surveillance in RNA functions. To investigate the mechanism by which cholinergic tone regulates hnRNPA2/B1 expression, we employed a combination of genetic mouse models and *in vivo* and *in vitro* techniques. Decreasing cholinergic tone reduced levels of hnRNPA2/B1, while increasing cholinergic signalling *in vivo* increased expression of hnRNPA2/B1. This effect is not due to decreased hnRNPA2/B1 mRNA expression, increased aggregation or degradation of the protein, but rather to decreased mRNA translation by nonsense mediated decay regulation of translation. Cell culture and knockout mice experiments demonstrated that M1 muscarinic signalling is critical for cholinergic control of hnRNPA2/B1 protein levels. Our experiments suggest an intricate regulation of hnRNPA2/B1 levels by cholinergic activity that interferes with alternative splicing in targeted neurons mimicking deficits found in AD.

6.2 Introduction

Dementia affects roughly 44 million individuals worldwide and represents a large economic burden (Wimo et al., 2013). Individuals affected with Alzheimer's disease (AD) present a profound decrease in basal forebrain cholinergic neurons (Whitehouse et al., 1982). These findings led to the cholinergic hypothesis of AD and the use of cholinesterase inhibitors to mitigate cholinergic failure (Bartus et al., 1982). Recent work suggests that the use of cholinesterase inhibitors for one year in possible prodromal AD-affected individuals halved rates of hippocampal atrophy, suggesting an intricate relationship between cholinergic tone and neurodegeneration (Dubois et al., 2015).

Cholinergic tone can modulate signal processing by changing electrical properties of cells and by modulating intracellular signalling (Dajas-Bailador and Wonnacott, 2004; Soreq, 2015). In addition, it has become clear that cholinergic signalling can also regulate long-term gene expression, miRNAs (Shaked et al., 2009; Soreq, 2015) and alternative splicing (Berson et al., 2012; Kolisnyk et al., 2013a), all of which can modulate functional properties of cells (Blencowe, 2006; Novarino et al., 2014). A key protein that regulates splicing events in AD is the heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1, which is reduced in brains of AD patients due to cholinergic deficiency (Berson et al., 2012) and is reduced in mice with a conditional deletion of the Vesicular Acetylcholine Transporter in the forebrain (VAChT) (Kolisnyk et al., 2013a).

hnRNPs are a large family of proteins which package pre-mRNA into larger hnRNP particles (Dreyfuss et al., 1993). Each of the hnRNPs contain distinct RNA binding motifs, which allows them to exert their roles in pre-mRNA processing (Black, 2003; Weighardt et al., 1996). hnRNPA2/B1 is one of the major hnRNP isoforms in the brain regulating alternative splicing and the transport of mRNA to distal cellular processes (Han et al., 2010). Importantly, knockdown of hnRNPA2/B1 in vivo caused impairments in learning and memory (Berson et al., 2012). Taken together, these findings suggest a critical role for this RNA binding protein in neuronal function and cognition.

Previous work suggests that cholinergic tone can regulate levels of hnRNPA2/B1, however the mechanisms involved are unclear. Here we show that hnRNPA2/B1 is a cholinergic controlled splicing factor. M1 muscarinic receptor activity is critical for cholinergic regulation of hnRNPA2B1 translation. This work provides a new mechanism by which acetylcholine (ACh) can influence targeted neurons, leading to potential widespread changes in neuronal function.

6.3 Materials and Methods

6.3.1 Mouse lines

All animals with targeted elimination of VAChT were generated using the VAChTflox/flox mouse described in (Martins-Silva et al., 2011). To eliminate VAChT from the forebrain, VAChTflox/flox mice were crossed with C57BL/6J-Tg(Nkx2-1-cre)2Sand/J mice (Xu et al., 2008) to generate VAChTNkx2.1-Cre-flox/flox (Al-Onaizi et al., 2016). To eliminate VAChT from the striatum the VAChTflox/flox mice were crossed with D2-Cre mice (Drd2, Line ER44) to generate VAChTD2-Cre-flox/flox (Guzman et al., 2011). VAChT overexpressing, ChAT-ChR2-EYFP mice were from the Jackson Laboratory, B6.Cg-Tg(Chat-Cop4*H134R/EYFP)6Gfng/J (Zhao et al., 2011). Generation of TgR (Shaked et al., 2009), M1-KO (Hamilton et al., 1997) and M4-KO mice (Gomeza et al., 1999) were previously described. All procedures were conducted in accordance with guidelines of the Canadian Council of Animal Care (CCAC) at the University of Western Ontario with an approved institutional animal protocol (2008-127). Only male mice were used for all experiments.

6.3.2 Immunofluorescence

Immunofluorescence experiments were performed as previously described (Guzman et al., 2011). Primary antibodies used were anti-hnRNPA2/B1 (1:200 Santa Cruz Biotechnology Catalog no. sc-10035), anti-NeuN (1:200 PhosphoSolutions, Catalog no.

583-FOX3), and anti-GFAP (1:500 AbCam, Catalogue no. ab7260). Sections were visualized by Zeiss LSM 510 Meta (Carl Zeiss, Oberkochen, Germany) confocal system (10x, 40x, 63x objectives), a 488-nm Ar laser and 633-nm HeNe laser were used for excitation of fluorophores.

6.3.3 Western Blotting

Mouse hippocampi were collected, proteins were isolated, and immunoblotting was performed as previously described using RIPA lysis buffer supplemented with protease and phosphatase inhibitors (Guzman et al., 2011). Band intensities were quantified using FluoroChemQ software (Thermo Fisher Scientific).

6.3.4Subcellular Fractionation

To isolate nuclear and cytoplasmic proteins from hippocampal tissue, the NE-PER Nuclear Protein Extraction Kit (Thermo Scientific, USA) was used following the manufacturer's instructions.

6.3.5 Sarkosyl Insolubility

Isolation of Sarkosyl insoluble protein was performed as described (Bai et al., 2013). Hippocampal tissue was homogenized in a low salt buffer (10 mM Tris, pH 7.5, 5 mM EDTA, 1 mM DTT, 10% (wt/vol) sucrose, Sigma protease inhibitor cocktail, ~10 ml buffer per gram tissue) to detect total protein, a detergent buffer (the low salt buffer with the addition of 1% (wt/vol) sarkosyl, N-Lauroylsarcosine) for the sarkosyl soluble fraction, and finally 8 M urea with 2% (wt/vol) SDS for the sorkosyl insoluble fraction.

6.3.6 Ubiquitination Assay

The ubiquitination status of the hnRNPA2/B1 protein was determined using previously described protocols (Choo and Zhang, 2009). Briefly, the hnRNPA2/B1 protein was immunoprecipitated using the Santa Cruz sc-10035 antibody and run on an SDS-Page gel. The gel was then blotted with an anti-ubiquitin antibody (Abcam ab7780).

6.3.7 Stereotaxic injections of adeno-associated virus

Injection of AAV virus has been described (Al-Onaizi et al., 2016). Briefly, mice were anesthetized with ketamine (100 mg/kg) and xylazine (25 mg/kg, and 1 µl (titer of ~1013 GC/ml) of AAV8-GFP-Cre-GFP or control virus (AAV8-GFP, Vector BioLabs, Eagleville, PA, USA) was injected into the medial septum (0.98 AP, 0.1 LL and 4.1 DV) of VAChTflox/flox mice. A recovery period of 4 weeks was given to allow transgene expression, prior to subsequent analyses.

6.3.8 RNA Sequencing

Total RNA was extracted from hippocampal tissues. cDNA library was prepared using TruSeq Stranded Total Sample Preparation kit (Illumina) and ran in a HiSeq 2500 platform. Datasets are available on ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-3897. Full description and analysis of the results of the RNA-Sequencing dataset are found in chapter 5.

6.3.9 qPCR and RT-PCR

To measure mRNA expression, total RNA was extracted from freshly dissected hippocampal tissue, using the Aurum Total RNA for fatty and fibrous tissue kit (Bio-Rad) according to the manufacturer's instructions. cDNA synthesis and qPCR analysis were performed as previously described (Guzman et al., 2011). For alternative splicing experiments, the alternative exon levels were normalized to a constitutively expressed exon from the same gene. RT-PCR was performed as previously described (Ribeiro et al., 2007).

6.3.10 Isolation of Polysomal RNA

Isolation of polysomal RNA was performed as described (Wagnon et al., 2012). Samples were homogenized in 1.5 ml of ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4, 3 mM MgCl2, 10 mM NaCl, 2% sucrose, 0.3% Triton X-100, 2 mM vanadyl ribonucleoside complexes [VRC] supplemented with protease inhibitors (complete mini, EDTA-free,

Roche, Indianapolis, IN). The homogenate was then centrifuged at 10,000 g for 10 minutes at 4°C, and the supernatant was transferred to a fresh tube. The lysate was treated with either 30 mM EDTA or 0.1 mg/ml RNase A for 30 minutes on ice or if they were to be treated with EDTA, VRC was not included in the lysis buffer. Lysates were then carefully layered onto 15–55% of sucrose in 25 mM Tris-HCl, pH 7.4, 25 mM NaCl, 5 mM MgCl2 (and 30 mM EDTA for the EDTA Samples). Gradients were ultracentrifuged in a Beckman Instruments SW40Ti rotor at 150,000g for 2 hours and 25 minutes at 4°C.

To isolate RNA from the fractions, RNA was collected into 1.5 ml of 7.7 M guanidine-HCl, 2 ml of 100% ethanol was added, and samples were stored at −20°C. Samples were then centrifuged at 4000 for 50 minutes at 4°C. The supernatant was removed and 200 µL DEPC-treated H2O was added to the pellet. To precipitate the RNA, 500 µL of 100% ethanol, 20 µL of 3 M sodium acetate, pH 5.2, and 10 µg glycogen were added, and the samples were stored overnight at −20°C. The samples were then centrifuged at 14,000g for 30 minutes at 4°C. Pellets were washed with ice-cold 75% ethanol and air dried for 15 minutes at room temperature. RNA was re-suspended in 30 µL DEPC-treated H2O and quantified by nanodrop, before being converted into cDNA using the Applied biosystems cDNA conversion kit. RT-PCR was then performed using primers designed to amplify hnRNPA2/B1 or β-Actin.

6.3.11Primary Neuronal Cultures

Primary cultures of hippocampal neurons from E17 mouse embryos were obtained as described previously (Beraldo et al., 2013). Cultures were maintained in Neurobasal medium with 2% B-27 supplement (Invitrogen). On day 4, cytosine arabinoside (2 µM; Sigma) was added to prevent astrocyte growth. Half of the culture medium was changed every 2–3 days. Neurons were cultured for 15 days

6.3.12 Pharmacological manipulations in Primary Neuronal Cultures

On the 15th day of culture, the neurons were treated with different doses of carbachol (0, 5, 10, or 50μM) dissolved in saline and, 48 hours later, total protein was isolated and levels of hnRNPA2/B1 were determined by Western blotting and immunofluorescence. To evaluate the contribution of nicotinic and muscarinic receptors in regulating hnRNPA2/B1 protein levels, neurons were pre-treated with either mechamylamine (100 μM), atropine (100 μM), or both. Then, 1 hour later, neurons were treated with carbachol (10 μM). Finally, to assess the effect of M1 muscarinic activation, neurons were treated with different doses of AF102B (0, 10, 100, 500 μM) dissolved in saline.

6.3.13 Statistical Analysis

Data are presented as mean ± SEM, unless otherwise stated. GraphPad Prism 6 software was used for statistical analysis. Comparison between two experimental groups was done by Student's t test. When several experimental groups or treatments were analyzed, one-way ANOVA and, when appropriate, a Tukey's HSD post hoc comparison test was used.

6.4 Results

6.4.1Cholinergic Modulation of hnRNPA2/B1 Protein Levels

Previous experiments indicated that hnRNPA2/B1 is decreased in the AD brains, but this is not modeled in genetic mouse models of AD (Berson et al., 2012). In contrast, either genetic or immunotoxin disruption of cholinergic tone led to decreased expression of hnRNP A2/B1 (Berson et al., 2012; Kolisnyk et al., 2013a). As expected VAChTNkx2.1-Cre-flox/flox animals presented a robust decrease in hnRNPA2/B1 levels in the hippocampus (Fig. 6.1 A).

The Nk2.1 promoter turns on Cre expression early during development (Xu et al., 2008) and therefore the resulting decrease in hnRNPA2/B1 levels could potentially be a result of developmental suppression of cholinergic tone, rather than being cholinergic regulated in adult mice. To test this possibility, we deleted the VAChT gene specifically in medial septum neurons and parts of the diagonal band (Al-Onaizi et al., 2016), which provides most of the hippocampal cholinergic innervation, of adult VAChTflox/flox mice using AAV8-Cre virus. AAV8-Cre-injected mice showed inter-related decline of both VAChT and hnRNPA2/B1 proteins, whereas AAV8-GFP-injected mice did not (Fig. 6.1B). There was a significant relationship between VAChT and hnRNP A2/B1 levels ($r^2=0.755$, $p<0.001$) (Fig. 6.1B). In addition, we tested whether, in the striatum, elimination of cholinergic tone would affect hnRNPA2/B1 expression by using VAChTD2-Cre-flox/flox mice, with selective striatal VAChT deficiency (Guzman et al., 2011). These mice showed no changes in hnRNPA2/B1 in their striatum (Fig. 6.1C), indicating hippocampal specificity of these effects.

If expression of hnRNPA2/B1 is a cholinergic-regulated process, one would expect that increased cholinergic tone should have opposite effects than those observed by decreased VAChT expression. ChAT-ChR2-EGFP mice overexpress VAChT in the hippocampus and consequently present increased cholinergic tone and ACh release (Kolisnyk et al., 2013b). These mice present increased hnRNPA2/B1 protein levels (Fig. 6.1D), suggesting that VAChT levels critically regulate hnRNPA2/B1 expression.

Decline of hnRNPA2/B1 could be solely related to VAChT depletion, or alternatively, it could reflect the functional loss of ACh signalling in the CNS. To distinguish between these possibilities, we tested the hippocampus of TgR mice over-expressing soluble AChE (Shaked et al., 2009), which causes cholinergic insufficiency. TgR mice showed decrease in hnRNPA2/B1 levels similar to mice with decreased VAChT (Fig. 6.1E), suggesting that ACh synaptic levels are causally involved in the regulation of hnRNPA2/B1 expression. Cre expression by itself had no effect on hnRNPA2/B1 levels as can be seen when we compared expression of hnRNPA2/B1 in the hippocampus of Nkx2.1-Cre and WT mice (Fig. 6.1F). Taken together these data give strong support for the hypothesis that hnRNPA2/B1 is a cholinergic regulated splicing factor.

RNA binding proteins such as hnRNPA2/B1 are predominantly expressed in the nucleus, but they can accumulate in the cytoplasm and cause neuronal toxicity (Kim et al., 2013; Wolozin, 2012). We therefore determined by immunofluorescence staining if the localization of hnRNP A2/B1 is changed in response to decreased cholinergic tone. Compared to controls VAChTNkx2.1-Cre-flox/flox had a decrease in hnRNPA2/B1 immunostaining in the CA1, CA3, and Dentate Gyrus regions of the hippocampus (Fig 6.2A-D). hnRNPA2/B1 was present mainly in the nucleus of both neurons (labeled by NeuN, Fig. 2-A-D) as well as in astrocytes (Fig. 6.2E) in control mice. We did not observe any shift in the localization of hnRNPA2/B1 in VAChT-deficient mice, only an overall decrease in the levels of staining. To confirm these observations we used subcellular fractionation to assess if VAChTNkx2.1-Cre-flox/flox mice show changes in hnRNPA2/B1 distribution between the nuclear and cytoplasmic fractions (Fig. 6.2F). In both control and VAChT-deficient mice, hnRNPA2/B1 was predominantly nuclear, following the distribution of the nuclear enzyme PIAS1 (Soares et al., 2013). However, VAChTNkx2.1-Cre-flox/flox mice showed consistently reduced nuclear hnRNPA2/B1 levels (Fig. 6.2F).

Figure 6.1. Analysis of hnRNPA2/B1 protein levels in genetically modified mice with differential expression of VAChT. (A) Representative Western blot and quantification of hnRNPA2/B1 protein expression in the hippocampus of $VAChT^{flox/flox}$ and $VAChT^{NKx2.1\text{-}Cre\text{-}flox/flox}$ mice. hnRNPA2/B1 expression was normalized to actin (n= 6). (B) hnRNPA2/B1 protein levels positively correlate with AAV induced reduction of VAChT. The graph shows values for each individual mice injected either with GFP or CRE. The image is from the medial septum of a virus-injected mice (C) Striatal elimination of VAChT does not alter hnRNPA2/B1 protein levels in the striatum of $VAChT^{D2\text{-}Cre\text{-}flox/flox}$ mice (n=4). (D) Transgenic mice over-expressing VAChT have increased hnRNPA2/B1 protein levels compared to controls (n=4). (E) hnRNPA2/B1 protein levels from the hippocampus of TgR transgenic mice (n=3). (F) No change in hnRNPA2/B1 protein levels compared to controls in the hippocampus of c567BL/6J-Nkx2.1-Cre mice (n=3) (Data are mean +/- S.E.M., *$p<0.05$, **$p<0.01$).

Figure 6.1. Analysis of hnRNPA2/B1 protein levels in genetically modified mice with differential expression of VAChT.

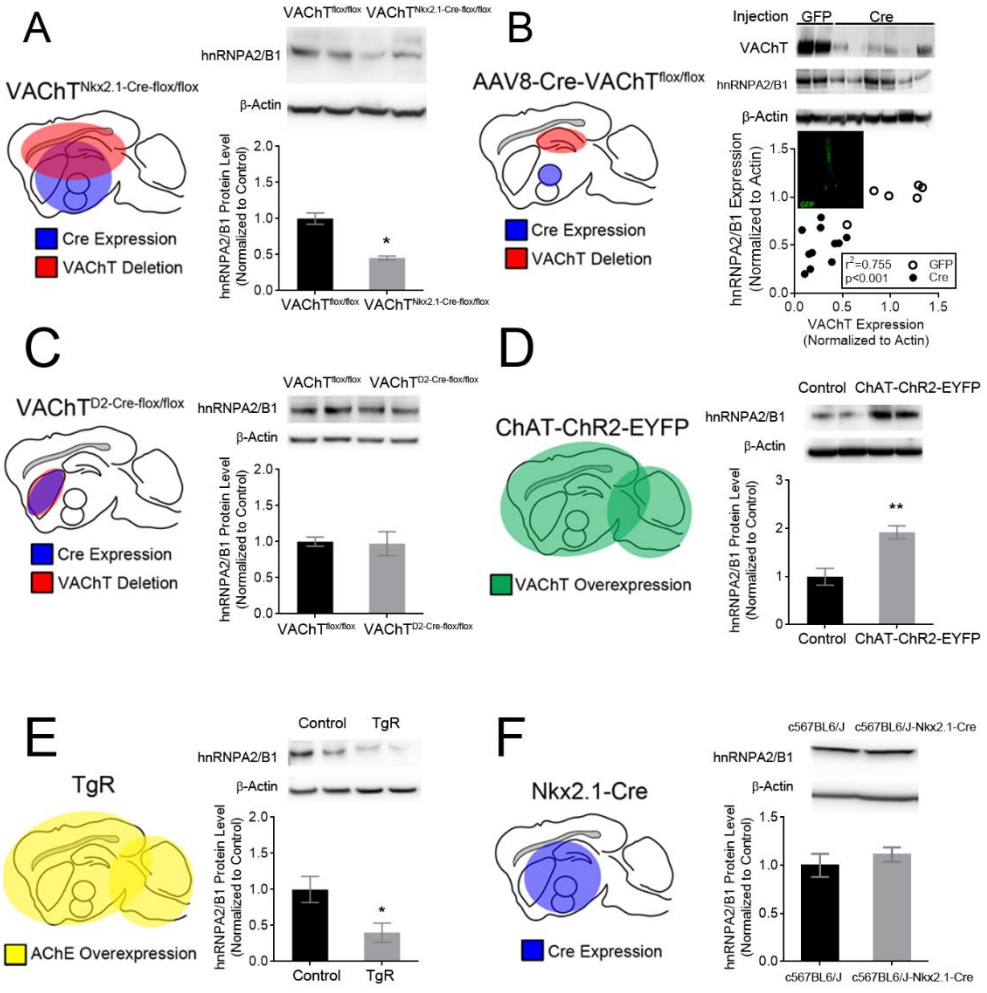
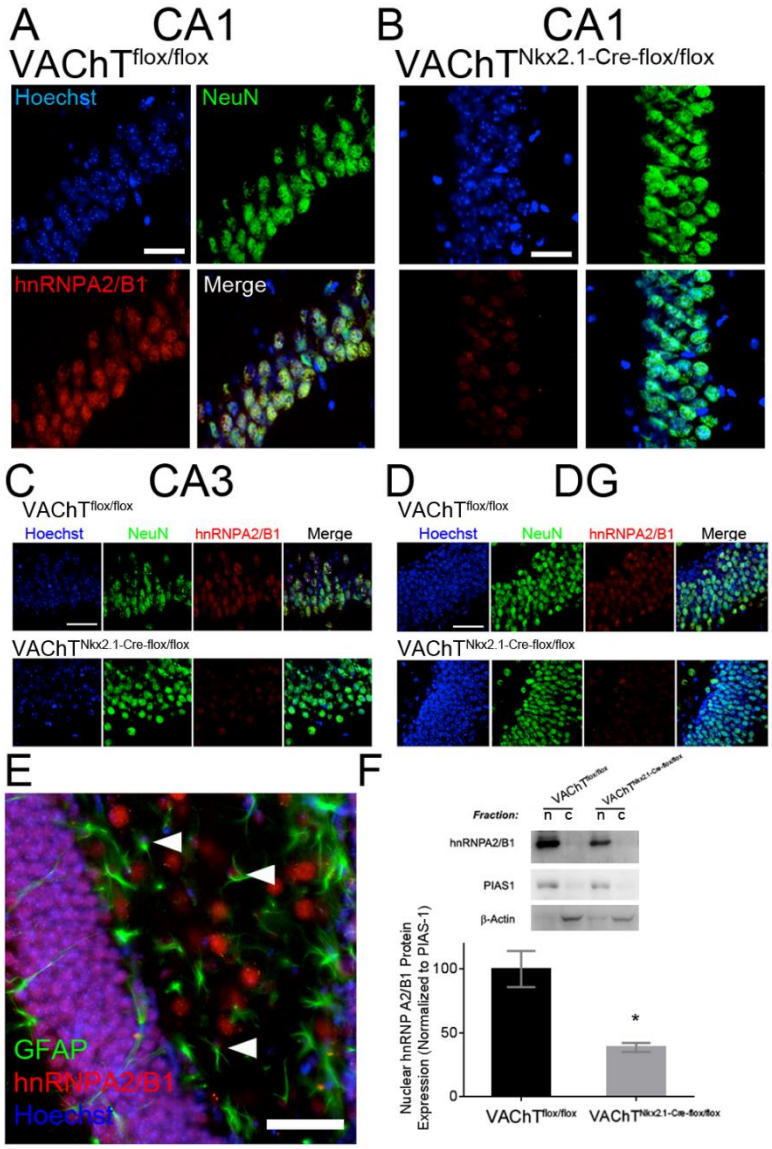


Figure 6.2. Characterization of decreased hnRNPA2/B1 protein levels in the hippocampus of VAChT-deficient mice. (A) Representative images of staining for NeuN, hnRNPA2/B1 and Hoeschst in the CA1 region of the hippocampus in controls (A) and VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ mice (B) (Scale bar, 50 um). Expression of hnRNPA2/B1 in the CA3 (C) and dentate gyrus (D) by immunofluorescence reveals general decrease of the protein and nuclear localization in VAChT-deficient mice. (E) Localization of hnRNPA2/B1 with GFAP glial marker in the hippocampus of VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice. (F) Subcellular fractionation assay of hnRNPA2/B1 protein shows hnRNPA2/B1 expression is mainly nuclear (data are mean +/- SEM, *P<0.05).

Figure 6.2. Characterization of decreased hnRNPA2/B1 protein levels in the hippocampus of VAChT-deficient mice.



6.4.2 Mechanisms of cholinergic modulation of hnRNPA2/B1

To investigate mechanisms by which cholinergic tone may regulate the levels of hnRNPA2/B1 protein, we evaluated ubiquitination, a modification that can facilitate protein degradation by the proteasome (Hochstrasser, 1996). Immunoprecipitated hnRNPA2/B1 from the hippocampus of controls and VAChTNkx2.1-Cre-flox/flox mice was resolved by SDS-PAGE and probed with ubiquitin antibodies. VAChT-deficient mice showed no change in ubiquitination status of hnRNPA2/B1 protein, when normalized to total hnRNPA2/B1 protein levels (Fig. 6.3A).

We also examined whether cholinergic tone affects aggregation of hnRNPA2/B1. Notably, hnRNPA2/B1 has a prion-like domain that favours increased aggregation when mutated (Kim et al., 2013). Protein aggregation was investigated by fractionating hippocampal extracts into sarkosyl soluble and insoluble fractions (Fig. 6.3B). In both controls and VAChT-deficient mice, hnRNPA2/B1 was mainly present in soluble fractions, unlike the U1-70k snRNP which has been shown to be present in insoluble fractions (Bai et al., 2013) (Fig. 6.3B). This result excluded the option that cholinergic tone increases aggregation of hnRNPA2/B1. Interestingly, despite different levels of hnRNPA2/B1 protein expression, both control and VAChT-deficient mice exhibited similar hnRNPA2/B1 mRNA levels as determined by RNA-Sequencing (Fig. 6.3C), as observed in AD brains (Berson et al., 2012).

It has been previously reported that protein levels of hnRNPA2/B1 are directly proportional to changes in the RNA editing of the 3’ UTR of its mRNA, with a shift away from a nonsense mediated decay (NMD) sensitive transcript increasing protein levels (Bonomi et al., 2013) (Fig. 6.3D). We evaluated by qPCR the ratio of NMD sensitive to NMD insensitive versions of the hnRNPA2/B1 transcript in the hippocampus of VAChTNkx2.1-Cre-flox/flox mice. Compared to controls, we observed a significant shift towards the NMD+ product in VAChT-deficient mice (Fig. 6.3E).

We then tested whether cholinergic tone modulation of NMD+ transcript could regulate hnRNPA2/B1 protein expression levels by limiting protein translation. For this, we studied the recruitment of hnRNPA2/B1 mRNA to ribosomes. Ribonucleotide-protein

complexes (RNPs) were isolated from hippocampal lysates, and sucrose density gradient fractionation was used to separate polyribosomes from large neuronal RNA granules (Fig. 6.4A) (Wagnon et al., 2012). Distribution of hnRNPA2/B1 transcripts in individual fractions was determined by RT–PCR in three individual mice of each genotype (Fig. 6.4B-C). In control mice, hnRNPA2/B1 mRNAs associated with polysomes and RNA granules, and treatment with EDTA, which dissociates mRNA from polysomes, equally distributed hnRNPA2/B1 mRNAs across fractions (Fig. 6.4D-E). However, in VAChTNkx2.1-Cre-flox/flox mice, distribution of hnRNPA2/B1 mRNAs was widespread throughout the fractions, resembling the distribution observed after EDTA treatment (Fig. 6.4D-E). The abundant β-actin mRNA remained unaltered between genotypes, demonstrating specificity towards hnRNPA2/B1 (Fig. 6.3F-G). These results indicate that decreased cholinergic tone leads to diminished translational capacity of hnRNPA2/B1 mRNA transcripts to modulate the efficiency of hnRNPA2/B1 protein translation.

Figure 6.3. Mechanisms of cholinergic regulation of hnRNPA2/B1 protein levels. (A) No change in ubiquitination status of hnRNPA2/B1 was observed when hnRNPA2/B1 from the hippocampus of VAChT deficient mice (p= 0.3067, n=7 Control and n=6 $VAChT^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice) was immunoprecipitated using anti-hnRNPA2/B1 antibody and probed with anti-ubiquitin antibody. (B) Sarkosyl insolubility assay shows no aggregation of hnRNPA2/B1 in $VAChT^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice (n=4). (C) Transcript level of hnRNPA2/B1 in the RNA-Seq dataset (p=0.9124, FDR=1). (D) Cartoon of the alternative splicing in the 3'UTR of hnRNPA2/B1 transcripts (Figure adapted from (Bonomi et al., 2013), the FL transcript is predicted to be stable and undergo translation whereas the NMD sensitive transcript is not; primers used to assay this event are show in the schematic. (E) Increased in the proportion of NMD+ hnRNPA2/B1 transcripts in the hippocampus of $VAChT^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice (n=4, $p<0.01$).

Figure 6.3. Mechanisms of cholinergic regulation of hnRNPA2/B1 protein levels.

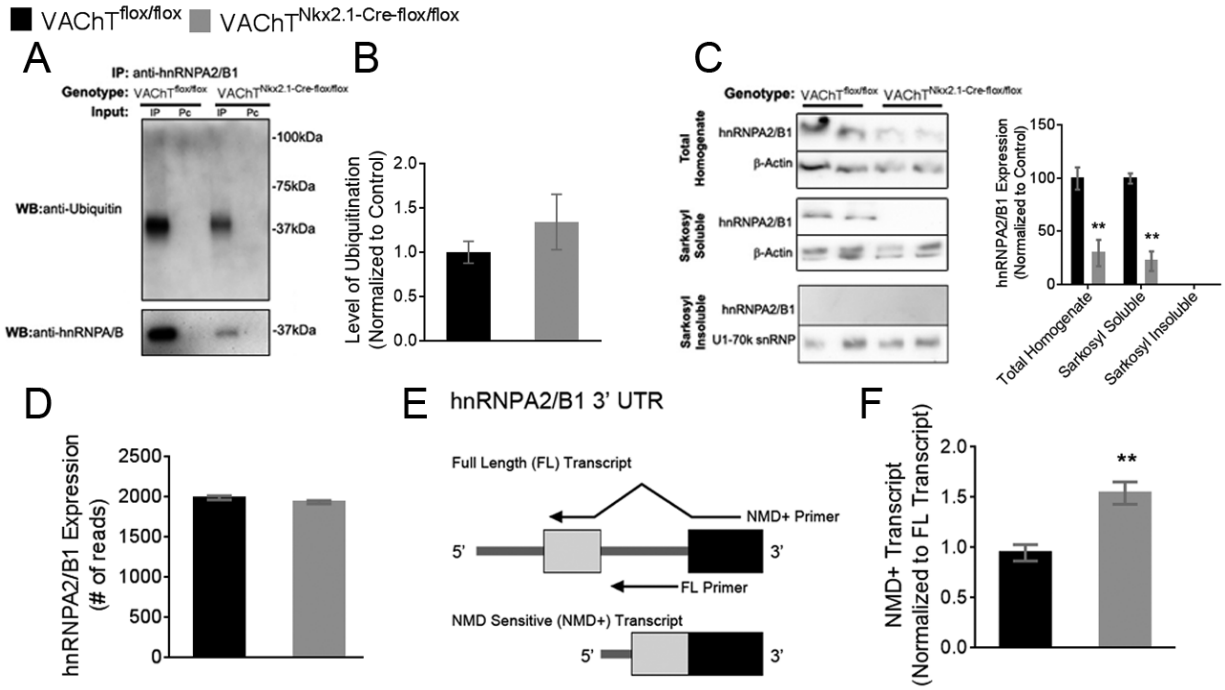
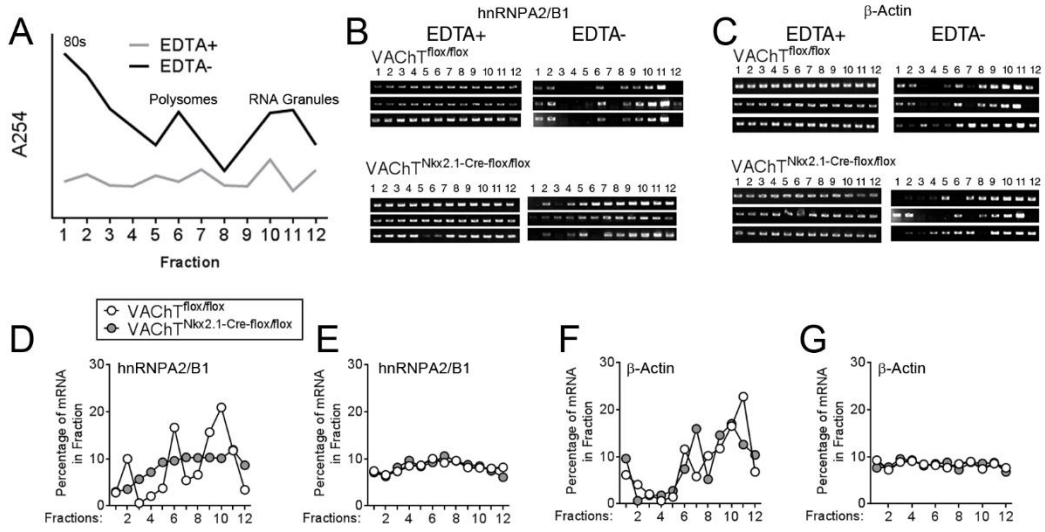


Figure 6.4. Forebrain cholinergic tone regulates translation of hNRNPA2/B1 in the hippocampus. (A) Hippocampal brain tissue was fractionated on a linear sucrose gradient. Fractions were collected and analyzed by spectrophotometry to determine position of monosome (80s), polysomes and RNA granules. (B) RT-PCR of hnRNPA2/B1 transcripts in VAChT$^{flox/flox}$ and VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice in the absence or presence of EDTA. (C) RT-PCR of β–Actin transcripts in VAChT$^{flox/flox}$ and VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice in the absence or presence of EDTA. (D-E) show quantification of data for the hnRNPA2/B1 transcripts from the three different VAChT-deficient and three control mice in the absence or presence of EDTA. (F-G) show quantification results for β–Actin transcripts from the three different VAChT-deficient and three control mice in the absence or presence of EDTA. 1-12 are the fraction numbers. The values plotted are averaged from gels in (C) (Data are mean).

Figure 6.4. Forebrain cholinergic tone regulates translation of hNRNPA2/B1 in the hippocampus.



6.4.3 Muscarinic Signalling Regulates hnRNPA2/B1 Translation by an NMD Mechanism

To further understand how cholinergic signalling regulates hnRNPA2/B1 levels, we treated neuronal hippocampal cultures from wild-type mice with the cholinergic mimetic carbachol (10μM) (Fig 6.5 A-B). This treatment effectively increased hnRNPA2/B1 protein levels in immunofluorescence and immunoblot experiments, and this effect could be blocked by muscarinic, but not by nicotinic antagonist treatment (Fig. 6.5C).

To study the contribution of muscarinic receptor subtypes, we evaluated hnRNPA2/B1 levels in the hippocampus of muscarinic receptor knockout mice. Compared to wild-type mice, M1, but not M4 receptor knockout mice, showed a decrease in hnRNPA2/B1 protein levels, resembling VAChTNkx2.1-Cre-flox/flox mice (Fig. 6.5D). Taken together, these experiments suggest that decreased cholinergic tone, likely due to insufficient M1 receptor activation, changes hnRNPA2/B1 protein levels by regulating mRNA translation.

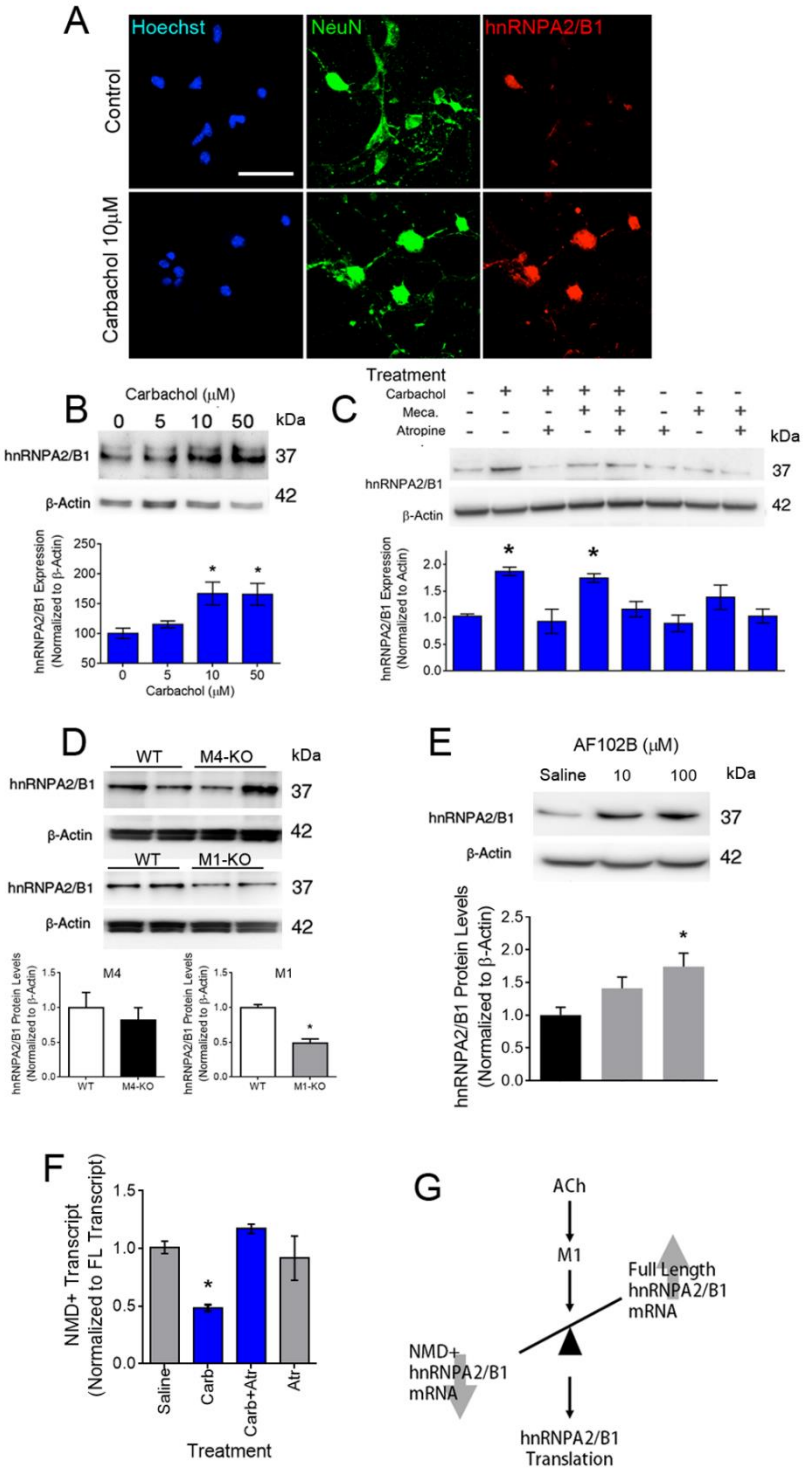
To further investigate the importance of M1 muscarinic receptors in the regulation of hnRNPA2/B1 protein levels in hippocampal neurons we treated neuronal hippocampal cultures from wild-type mice with the M1 muscarinic agonist AF102B (Fisher et al., 1989). Compared to saline treated neurons, those treated with 100 μM of AF102B showed a significant increase in protein levels of hnRNPA2/B1 (Fig. 6.5E). Interestingly, neurons treated with 500 μM of the compound did not show this effect, suggesting a “U-shaped” dose-effect function for this effect, a reported effect for M1 muscarinic receptors (Thomsen et al., 2012).

We then evaluated whether in vitro cholinergic regulation also changes the NMD+ product. Compared to saline treatment, carbachol shifted the expression of hnRNPA2/B1 RNA towards the NMD insensitive full-length mRNA product (Fig 6.5F). Furthermore co-treatment with atropine blocked this effect and returned the ratio to control levels, similar to what we observed for hnRNPA2/B1 protein levels (Fig. 6.5C). These data suggest that the ratio of NMD+ hnRNPA2/B1 gene products predict change

in protein levels, and that the regulation of NMD sensitivity is dependent on muscarinic signalling.

Figure 6.5. Muscarinic Regulation of hnRNPA2/B1 Translation. (A) Representative immunofluorescence images and (B) Western blots with quantification of hnRNPA2/B1 protein levels in primary hippocampal neurons after treatment with carbachol for 48 hours. hnRNPA2/B1 expression was normalized to actin (n=3, Data are mean ± SEM. *p<0.05). (C) Representative Western blot and quantification of hnRNPA2/B1 expression in primary hippocampal neurons after treatment with carbachol, mecamylamine, and atropine. hnRNPA2/B1 expression was normalized to actin (n=4, *p<0.05). The values plotted are averaged from the gels of each individual experiment. (D) Representative Western blot and quantification of hnRNPA2/B1 expression in hippocampal tissue of M4 muscarinic knockout mice (n=7), and M1 receptor knockout mice (n=7 Control and n= 8 M1 KO mice *p<0.05). (E) M1 muscarinic receptor agonist AF102B significantly increased hnRNPA2/B1 protein levels in primary hippocampal cultures (n=5 *p<0.05). (F) Increased expression of the full-length hnRNPA2/B1 transcripts of cells treated with carbachol is blocked by the administration of atropine (n=5, *p<0.05). (G) Proposed model wherein muscarinic M1 receptors signaling would shift the ratio of hnRNPA2/B transcripts, favouring the full length transcripts, and therefore increase translation of the protein.

Figure 6.5. Muscarinic Regulation of hnRNPA2/B1 Translation.



6.5 Discussion

In this study we combined a variety of in vivo and in vitro techniques to evaluate the contribution of cholinergic signalling to expression levels of hippocampal hnRNPA2/B1 protein. Using a number of mouse lines, we demonstrated that hnRNPA2/B1 protein levels in the hippocampus are exquisitely sensitive to changes in cholinergic tone.

Interestingly, we observed no change in hnRNPA2/B1 protein levels in striatum- specific VAChT mutants, whereas there is a body of evidence that cholinergic signalling can affect the levels of this protein in both cortical and hippocampal regions in vivo (Berson et al., 2012; Kolisnyk et al., 2013a). Given these findings it is likely that it is an intrinsic property of the target cells themselves that dictate their change in hnRNPA2/B1 translation in response to cholinergic activity. Our results highlight the critical role of the Gq coupled M1 muscarinic receptor in governing hnRNPA2/B1 protein levels.

Unlike the rare hnRNPA2/B1 mutation that increases aggregation and nuclear exclusion (Kim et al., 2013), we did not find aggregation of hnRNPA2/B1 in mice with forebrain cholinergic deficiency, suggesting that in these mice, and likely in AD brains, hnRNPA2/B1 dysfunction occurs by a separate and distinct mechanism. Furthermore, we did not see an increase in ubiquitination, suggesting that changes in hnRNPA2/B1 protein levels do not occur at the post-translational level. In line with this, we found that it may in fact be abnormal translation that drives regulation of hnRNPA2/B1.

A common mechanism for the regulation of the translation of RNA binding proteins is regulation by unproductive splicing and translation (RUST), where the alternative spicing of a transcript affects its translation efficiency (Lareau et al., 2007; McGlincy and Smith, 2008). This may serve as a potential mechanism for cholinergic control of the translation of this RBP. Accordingly, we found that the levels of hnRNPA2/B1 transcripts were maintained in cholinergic-deficient mice or AD brains (Berson et al., 2012), but hnRNPA2/B1 translation was selectively decreased. Notably, hnRNPA2/B1 has been shown to be auto-regulated by a RUST mechanism involving alternative splicing in its 3′-untranslated region that leads to NMD driven by mTOR1C (Dempsey, 2012; McGlincy et al., 2010), which is a key effector of muscarinic receptor signalling (Slack

and Blusztajn, 2008). Correspondingly, we found that cholinergic control of hnRNPA2/B1 translation is mediated by M1 muscarinic receptors (Figure 6.5G).

Targeting the interactions between RBPs and RNA may serve as a new potential therapeutic avenue to restore the RNA-editing deficits observed in neurodegenerative diseases (Bai et al., 2013; Berson et al., 2012; Qian and Liu, 2014; Tollervey et al., 2011). A number of substances, including regularly prescribed antibiotics, have been shown to non-selectively alter alternative splicing in the brain (Graveley, 2005; Kole et al., 2012; Tollervey et al., 2011), however this approach lacks the ability to specifically target “impaired” RBP-RNA interactions.

Cholinergic failure is one of the hallmarks of AD, with the basal forebrain cholinergic system being heavily affected by the disease (Whitehouse et al., 1982). Data from the ADNI (Alzheimer’s Disease Neuroimaging Initiative) consortium, has linked cholinergic failure in AD to both pathological outcomes (Teipel et al., 2014) as well as cognitive impairments in AD (Grothe et al., 2014). Further evidence for the importance of cholinergic signalling to the etiology of AD comes from clinical evidence that the long-term use of anticholinergic medication, specifically anti-muscarinic drugs, significantly increases the risk of developing dementia (Gray et al., 2015). Interestingly, administration of anti-muscarinic agents to AD patients exacerbates their symptoms (Lim et al., 2015). Together these results suggest a crucial role for cholinergic tone in AD, with specific importance of muscarinic signalling.

The main pathological hallmarks of AD are the accumulation of Aβ plaques and of hyperphosphorylated tau (Huang and Jiang, 2009). M1 muscarinic signalling has been linked to both of these processes. Activation of the receptor has been shown to alter tau phosphorylation both in vitro (Sadot et al., 1996) and in vivo (Genis et al., 1999). Moreover, deletion of M1 muscarinic receptors increases Aβ related pathology in a transgenic mouse model overexpressing mutated APP (Davis et al., 2010). Furthermore, M1 agonists have been shown to reverse Aβ related pathology in mouse models of AD (Caccamo et al., 2006). These findings suggest that M1 receptors are key mediators of AD pathology. How hnRNPA2/B1 protein expression may contribute to pathology remains to be determined.

Cholinergic failure also plays an important role in one of the most important and apparent cognitive deficits of AD, memory loss (Bartus et al., 1982). There is a strong correlation between loss of basal forebrain cholinergic neurons and cognitive functioning in AD patients. Furthermore, mice with a forebrain specific deletion of VAChT have severe deficits in performance on the Paired-associates learning (PAL) touchscreen task (Al-Onaizi et al., 2016), a rodent version of the Cambridge Neuropsychological Test Automated Battery (CANTAB) tests used in humans, which has been shown to be selective for the memory impairments in AD patients (Egerhazi et al., 2007). Importantly, lentiviral mediated knockdown of hnRNPA2/B1 also produced cognition impairments in mice (Berson et al., 2012).

Taken together, our findings indicate an intricate relationship between M1 muscarinic signalling and hnRNPA2/B1 translation. These findings lay the ground work for new therapeutic avenues for the treatment of AD. Specifically, it points to the potential of M1 muscarinic positive allosteric modulators to improve long-term changes in RNA metabolism and cognitive deficits due to cholinergic malfunction in AD. Noteworthy M1 muscarinic positive allosteric modulators have shown promising results to improve cognition in non-human primates (Lange et al., 2015). Our results show novel mechanisms by which log-term cholinergic dysfunction can regulate target cells.

6.6 Acknowledgements

This work was supported by the Canadian Institute of Health Research (MOP 136930, MOP 126000 and MOP 89919), NSERC (402524-2013), Brain Canada, Canadian Foundation for Innovation, and Ontario research fund (M.A.M.P. and V.F.P.). H.S. acknowledges support by the European Research Council (Advanced Award 321501), and the Legacy Heritage Science Initiative (LHSI) of the Israel Science Foundation (Grant No. 378/11). B.K. is a recipient of the Annie Darkens Research Fund Award from the Alzheimer's Society of Canada fellowship, M.A.A-O received fellowship support from Kuwait University and G.M.P. was a recipient of a Science without borders fellowship from the Brazilian government.

6.7 References

Al-Onaizi, M.A., Parfitt, G.M., Kolisnyk, B., Law, C.S., Guzman, M.S., Barros, D.M., Leung, L.S., Prado, M.A., and Prado, V.F. (2016). Regulation of Cognitive Processing by Hippocampal Cholinergic Tone. Cereb Cortex.

Bai, B., Hales, C.M., Chen, P.C., Gozal, Y., Dammer, E.B., Fritz, J.J., Wang, X., Xia, Q., Duong, D.M., Street, C., et al. (2013). U1 small nuclear ribonucleoprotein complex and RNA splicing alterations in Alzheimer's disease. Proceedings of the National Academy of Sciences of the United States of America 110, 16562-16567.

Bartus, R.T., Dean, R.L., 3rd, Beer, B., and Lippa, A.S. (1982). The cholinergic hypothesis of geriatric memory dysfunction. Science 217, 408-414.

Beraldo, F.H., Soares, I.N., Goncalves, D.F., Fan, J., Thomas, A.A., Santos, T.G., Mohammad, A.H., Roffe, M., Calder, M.D., Nikolova, S., et al. (2013). Stress-inducible phosphoprotein 1 has unique cochaperone activity during development and regulates cellular response to ischemia via the prion protein. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 27, 3594-3607.

Berson, A., Barbash, S., Shaltiel, G., Goll, Y., Hanin, G., Greenberg, D.S., Ketzef, M., Becker, A.J., Friedman, A., and Soreq, H. (2012). Cholinergic-associated loss of hnRNP-A/B in Alzheimer's disease impairs cortical splicing and cognitive function in mice. EMBO Mol Med 4, 730-742.

Black, D.L. (2003). Mechanisms of alternative pre-messenger RNA splicing. Annual review of biochemistry 72, 291-336.

Blencowe, B.J. (2006). Alternative splicing: new insights from global analyses. Cell 126, 37-47.

Bonomi, S., di Matteo, A., Buratti, E., Cabianca, D.S., Baralle, F.E., Ghigna, C., and Biamonti, G. (2013). HnRNP A1 controls a splicing regulatory circuit promoting mesenchymal-to-epithelial transition. Nucleic acids research 41, 8665-8679.

Caccamo, A., Oddo, S., Billings, L.M., Green, K.N., Martinez-Coria, H., Fisher, A., and LaFerla, F.M. (2006). M1 receptors play a central role in modulating AD-like pathology in transgenic mice. Neuron 49, 671-682.

Choo, Y.S., and Zhang, Z. (2009). Detection of protein ubiquitination. Journal of visualized experiments : JoVE.

Dajas-Bailador, F., and Wonnacott, S. (2004). Nicotinic acetylcholine receptors and the regulation of neuronal signalling. Trends in pharmacological sciences 25, 317-324.

Davis, A.A., Fritz, J.J., Wess, J., Lah, J.J., and Levey, A.I. (2010). Deletion of M1 muscarinic acetylcholine receptors increases amyloid pathology in vitro and in vivo. The Journal of neuroscience : the official journal of the Society for Neuroscience 30, 4190-4196.

Dempsey, J. M. 2012. SRPK2 Phosphorylation by the AGC Kinases, and mTORC1 Regulation of Alternative Splicing. Doctoral dissertation, Harvard University.

Dreyfuss, G., Matunis, M.J., Pinol-Roma, S., and Burd, C.G. (1993). hnRNP proteins and the biogenesis of mRNA. Annual review of biochemistry 62, 289-321.

Dubois, B., Chupin, M., Hampel, H., Lista, S., Cavedo, E., Croisile, B., Louis Tisserand, G., Touchon, J., Bonafe, A., Ousset, P.J., et al. (2015). Donepezil decreases annual rate of hippocampal atrophy in suspected prodromal Alzheimer's disease. Alzheimer's & dementia : the journal of the Alzheimer's Association 11, 1041-1049.

Egerhazi, A., Berecz, R., Bartok, E., and Degrell, I. (2007). Automated Neuropsychological Test Battery (CANTAB) in mild cognitive impairment and in Alzheimer's disease. Progress in neuro-psychopharmacology & biological psychiatry 31, 746-751.

Fisher, A., Brandeis, R., Pittel, Z., Karton, I., Sapir, M., Dachir, S., Levy, A., and Heldman, E. (1989). (+-)-cis-2-methyl-spiro(1,3-oxathiolane-5,3') quinuclidine (AF102B): a new M1 agonist attenuates cognitive dysfunctions in AF64A-treated rats. Neuroscience letters 102, 325-331.

Genis, I., Fisher, A., and Michaelson, D.M. (1999). Site-specific dephosphorylation of tau of apolipoprotein E-deficient and control mice by M1 muscarinic agonist treatment. Journal of neurochemistry 72, 206-213.

Gomeza, J., Zhang, L., Kostenis, E., Felder, C., Bymaster, F., Brodkin, J., Shannon, H., Xia, B., Deng, C., and Wess, J. (1999). Enhancement of D1 dopamine receptor-mediated locomotor stimulation in M(4) muscarinic acetylcholine receptor knockout mice. Proceedings of the National Academy of Sciences of the United States of America 96, 10483-10488.

Graveley, B.R. (2005). Small molecule control of pre-mRNA splicing. RNA 11, 355-358.

Gray, S.L., Anderson, M.L., Dublin, S., Hanlon, J.T., Hubbard, R., Walker, R., Yu, O., Crane, P.K., and Larson, E.B. (2015). Cumulative use of strong anticholinergics and incident dementia: a prospective cohort study. JAMA internal medicine 175, 401-407.

Grothe, M.J., Ewers, M., Krause, B., Heinsen, H., Teipel, S.J., and Alzheimer's Disease Neuroimaging, I. (2014). Basal forebrain atrophy and cortical amyloid deposition in nondemented elderly subjects. Alzheimer's & dementia : the journal of the Alzheimer's Association 10, S344-353.

Guzman, M.S., De Jaeger, X., Raulic, S., Souza, I.A., Li, A.X., Schmid, S., Menon, R.S., Gainetdinov, R.R., Caron, M.G., Bartha, R., et al. (2011). Elimination of the vesicular acetylcholine transporter in the striatum reveals regulation of behaviour by cholinergic-glutamatergic co-transmission. PLoS biology 9, e1001194.

Hamilton, S.E., Loose, M.D., Qi, M., Levey, A.I., Hille, B., McKnight, G.S., Idzerda, R.L., and Nathanson, N.M. (1997). Disruption of the m1 receptor gene ablates muscarinic receptor-dependent M current regulation and seizure activity in mice. Proceedings of the National Academy of Sciences of the United States of America 94, 13311-13316.

Han, S.P., Friend, L.R., Carson, J.H., Korza, G., Barbarese, E., Maggipinto, M., Hatfield, J.T., Rothnagel, J.A., and Smith, R. (2010). Differential subcellular distributions and trafficking functions of hnRNP A2/B1 spliceoforms. Traffic 11, 886-898.

Hochstrasser, M. (1996). Ubiquitin-dependent protein degradation. Annual review of genetics 30, 405-439.

Huang, H.C., and Jiang, Z.F. (2009). Accumulated amyloid-beta peptide and hyperphosphorylated tau protein: relationship and links in Alzheimer's disease. Journal of Alzheimer's disease : JAD 16, 15-27.

Kim, H.J., Kim, N.C., Wang, Y.D., Scarborough, E.A., Moore, J., Diaz, Z., MacLea, K.S., Freibaum, B., Li, S., Molliex, A., et al. (2013). Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS. Nature 495, 467-473.

Kole, R., Krainer, A.R., and Altman, S. (2012). RNA therapeutics: beyond RNA interference and antisense oligonucleotides. Nature reviews Drug discovery 11, 125-140.

Kolisnyk, B., Al-Onaizi, M.A., Hirata, P.H., Guzman, M.S., Nikolova, S., Barbash, S., Soreq, H., Bartha, R., Prado, M.A., and Prado, V.F. (2013a). Forebrain deletion of the vesicular acetylcholine transporter results in deficits in executive function, metabolic, and RNA splicing abnormalities in the prefrontal cortex. The Journal of neuroscience : the official journal of the Society for Neuroscience 33, 14908-14920.

Kolisnyk, B., Guzman, M.S., Raulic, S., Fan, J., Magalhaes, A.C., Feng, G., Gros, R., Prado, V.F., and Prado, M.A. (2013b). ChAT-ChR2-EYFP mice have enhanced motor endurance but show deficits in attention and several additional cognitive domains. The Journal of neuroscience : the official journal of the Society for Neuroscience 33, 10427-10438.

Lange, H.S., Cannon, C.E., Drott, J.T., Kuduk, S.D., and Uslaner, J.M. (2015). The M1 Muscarinic Positive Allosteric Modulator PQCA Improves Performance on Translatable Tests of Memory and Attention in Rhesus Monkeys. The Journal of pharmacology and experimental therapeutics 355, 442-450.

Lareau, L.F., Brooks, A.N., Soergel, D.A., Meng, Q., and Brenner, S.E. (2007). The coupling of alternative splicing and nonsense-mediated mRNA decay. Advances in experimental medicine and biology 623, 190-211.

Lim, Y.Y., Maruff, P., Schindler, R., Ott, B.R., Salloway, S., Yoo, D.C., Noto, R.B., Santos, C.Y., and Snyder, P.J. (2015). Disruption of cholinergic neurotransmission exacerbates Abeta-related cognitive impairment in preclinical Alzheimer's disease. Neurobiology of aging 36, 2709-2715.

Martins-Silva, C., De Jaeger, X., Guzman, M.S., Lima, R.D., Santos, M.S., Kushmerick, C., Gomez, M.V., Caron, M.G., Prado, M.A., and Prado, V.F. (2011). Novel strains of mice deficient for the vesicular acetylcholine transporter: insights on transcriptional regulation and control of locomotor behavior. PloS one 6, e17611.

McGlincy, N.J., and Smith, C.W. (2008). Alternative splicing resulting in nonsense-mediated mRNA decay: what is the meaning of nonsense? Trends in biochemical sciences 33, 385-393.

McGlincy, N.J., Tan, L.Y., Paul, N., Zavolan, M., Lilley, K.S., and Smith, C.W. (2010). Expression proteomics of UPF1 knockdown in HeLa cells reveals autoregulation of hnRNP A2/B1 mediated by alternative splicing resulting in nonsense-mediated mRNA decay. BMC genomics 11, 565.

Novarino, G., Fenstermaker, A.G., Zaki, M.S., Hofree, M., Silhavy, J.L., Heiberg, A.D., Abdellateef, M., Rosti, B., Scott, E., Mansour, L., et al. (2014). Exome sequencing links corticospinal motor neuron disease to common neurodegenerative disorders. Science 343, 506-511.

Qian, W., and Liu, F. (2014). Regulation of alternative splicing of tau exon 10. Neuroscience bulletin 30, 367-377.

Ribeiro, F.M., Ferreira, L.T., Marion, S., Fontes, S., Gomez, M., Ferguson, S.S., Prado, M.A., and Prado, V.F. (2007). SEC14-like protein 1 interacts with cholinergic transporters. Neurochemistry international 50, 356-364.

Sadot, E., Gurwitz, D., Barg, J., Behar, L., Ginzburg, I., and Fisher, A. (1996). Activation of m1 muscarinic acetylcholine receptor regulates tau phosphorylation in transfected PC12 cells. Journal of neurochemistry 66, 877-880.

Shaked, I., Meerson, A., Wolf, Y., Avni, R., Greenberg, D., Gilboa-Geffen, A., and Soreq, H. (2009). MicroRNA-132 potentiates cholinergic anti-inflammatory signaling by targeting acetylcholinesterase. Immunity 31, 965-973.

Slack, B.E., and Blusztajn, J.K. (2008). Differential regulation of mTOR-dependent S6 phosphorylation by muscarinic acetylcholine receptor subtypes. Journal of cellular biochemistry 104, 1818-1831.

Soares, I.N., Caetano, F.A., Pinder, J., Rodrigues, B.R., Beraldo, F.H., Ostapchenko, V.G., Durette, C., Pereira, G.S., Lopes, M.H., Queiroz-Hazarbassanov, N., et al. (2013). Regulation of stress-inducible phosphoprotein 1 nuclear retention by protein inhibitor of activated STAT PIAS1. Molecular & cellular proteomics : MCP 12, 3253-3270.

Soreq, H. (2015). Checks and balances on cholinergic signaling in brain and body function. Trends in neurosciences 38, 448-458.

Teipel, S., Heinsen, H., Amaro, E., Jr., Grinberg, L.T., Krause, B., Grothe, M., and Alzheimer's Disease Neuroimaging, I. (2014). Cholinergic basal forebrain atrophy predicts amyloid burden in Alzheimer's disease. Neurobiol Aging 35, 482-491.

Thomsen, M., Lindsley, C.W., Conn, P.J., Wessell, J.E., Fulton, B.S., Wess, J., and Caine, S.B. (2012). Contribution of both M1 and M4 receptors to muscarinic agonist-mediated attenuation of the cocaine discriminative stimulus in mice. Psychopharmacology 220, 673-685.

Tollervey, J.R., Wang, Z., Hortobagyi, T., Witten, J.T., Zarnack, K., Kayikci, M., Clark, T.A., Schweitzer, A.C., Rot, G., Curk, T., et al. (2011). Analysis of alternative splicing associated with aging and neurodegeneration in the human brain. Genome research 21, 1572-1582.

Wagnon, J.L., Briese, M., Sun, W., Mahaffey, C.L., Curk, T., Rot, G., Ule, J., and Frankel, W.N. (2012). CELF4 regulates translation and local abundance of a vast set of

mRNAs, including genes associated with regulation of synaptic function. PLoS Genet 8, e1003067.

Weighardt, F., Biamonti, G., and Riva, S. (1996). The roles of heterogeneous nuclear ribonucleoproteins (hnRNP) in RNA metabolism. BioEssays : news and reviews in molecular, cellular and developmental biology 18, 747-756.

Whitehouse, P.J., Price, D.L., Struble, R.G., Clark, A.W., Coyle, J.T., and Delon, M.R. (1982). Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. Science 215, 1237-1239.

Wimo, A., Jonsson, L., Bond, J., Prince, M., and Winblad, B. (2013). The worldwide economic impact of dementia 2010. Alzheimer's & dementia : the journal of the Alzheimer's Association 9, 1-11 e13.

Wolozin, B. (2012). Regulated protein aggregation: stress granules and neurodegeneration. Molecular neurodegeneration 7, 56.

Xu, Q., Tam, M., and Anderson, S.A. (2008). Fate mapping Nkx2.1-lineage cells in the mouse telencephalon. The Journal of comparative neurology 506, 16-29.

Zhao, S., Ting, J.T., Atallah, H.E., Qiu, L., Tan, J., Gloss, B., Augustine, G.J., Deisseroth, K., Luo, M., Graybiel, A.M., and Feng, G. (2011). Cell type-specific channelrhodopsin-2 transgenic mice for optogenetic dissection of neural circuitry function. Nature methods 8, 745-752.

7 Chapter 7

Summary and Conclusion

7.1 Summary of Major Findings

Cholinergic dysfunction is a hallmark of Alzheimer's disease. This dysfunction is hypothesized to underlie cognitive symptoms of the disease. However, the exact contribution of cholinergic dysfunction to the etiology of the disease is unclear. In this thesis, we sought out to determine whether alterations in cholinergic signalling in the brain (either increased or decreased) leads to changes in cognitive functioning. Furthermore our goal was to determine the molecular mechanisms of cholinergic dysfunction, and how changes in these mechanisms can contribute to the pathological outcomes in Alzheimer's disease.

In chapter 2 of this thesis, we characterized a mouse line which carries several copies of the vesicular acetylcholine transporter (*VAChT*) gene. We showed that increase in VAChT gene copy number leads to overexpression of functional *VAChT* with consequent increase in cholinergic tone. We then carried out a series of behavioural assays to determine the implications on cognition of increased cholinergic tone. We demonstrated that these mice have marked improvement in motor endurance. However, they also have severe cognitive deficits, including attention deficits and dysfunction in working memory and spatial memory. These data, taken together, showed that increased VAChT expression increases acetylcholine release in the brain and that this is severely detrimental to the cognitive processing of the animals. Although cholinergic tone is reduced in the Alzheimer's brain, the data presented highlight the importance of striking a balance in cholinergic signalling in the brain.

In chapter 3, we endeavoured to evaluate the effect of long-term cholinergic dysfunction in the forebrain. We evaluated the involvement of acetylcholine to forebrain function by genetically eliminating VAChT from this population of neurons. Included in the

acetylcholine innervated forebrain regions is the PFC, a key brain region in the regulation of executive functioning. We therefore tested mice on measures of executive function. This was assessed using both a pairwise visual discrimination test and a 5-choice serial reaction time task (5-CSRT). Results of the pairwise test showed that VAChT-mutant mice were able to learn the initial stimulus pairing, however when the stimulus pairings were switched the mice failed to learn the new stimulus-reward rule. Similarly, on the 5-CSRT the VAChT mutant animals were able to learn the task, but once the stimulus presentation length was reduced (increasing attentional demand), the mice showed prominent impairments on the task compared to controls.

Given the impaired cognition of the animals, we performed *in vivo* magnetic resonance spectroscopy, to assess potential changes in PFC circuitry and neuronal function. This analysis showed changed levels of the metabolites taurine and lactate. The results suggested changes in neuronal metabolism in the PFC of the VAChT-deficient mice. The PFC of the mice showed a severe decrease in the protein levels of the RNA binding protein heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNPA2/B1). Accordingly, a number of genes in the PFC of VAChT-deficient mice were found to be alternatively spliced. Amongst these genes was pyruvate kinase M, a key enzyme involved in lactate metabolism. In chapter 3, we were able to determine the impact of cholinergic dysfunction in the forebrain and furthermore were able to determine some of the molecular mechanisms that result from cholinergic dysfunction. We therefore sought to expand upon these two key findings in the following chapters.

Chapter 4 was specifically designed to dissect the mechanism underlying cholinergic control of attention, based on our findings in chapter 3 that mice lacking release of acetylcholine into the PFC were impaired on a task of attention. Chapter 4 was dedicated to determining the roles of nicotinic cholinergic receptors in this task. Significant literature exists examining the role of β2 nicotinic receptors (b2nAChR) on the 5-CSRT, however, the role of the α7 nicotinic ACh receptor (α7nAChR) in attention is ambiguous. To clarify the role of the receptor on the task, we trained α7nAChR knockout mice on the 5-CSRT task. α7nAChR knockout mice showed impairments in

measures of sustained attention. This was assessed by comparing the performance on the first half of the task to the second half. We were able to completely reverse these impairments by treating mutant mice with an agonist of the β2nAChR. In addition, treating α7nAChR knockout mice with β2nAChR agonist activated the same biochemical response in the PFC as did the administration of a α7nAChR agonists in control, non-transgenic mice. The work presented in chapter 4 of this thesis details a complex interplay between the α7nAChR and β2nAChR receptors. This complex relationship regulates attentional performance on the 5CSRT task in mice. These data provide detailed mechanistic insight into cholinergic regulation of attention.

In chapter 5, we pursued the specific molecular mechanisms that underlie long-term cholinergic dysfunction in the brain. To achieve this, we used mice with a deletion of VAChT in the forebrain in order to model cholinergic aberrations. We then employed RNA-sequencing of hippocampal samples from these mice to study genome-wide transcriptome changes. Using this approach we were able to show that cholinergic dysfunction produces alterations in RNA metabolism. We then determined the impact of these changes in RNA metabolism by focusing our studies on changes in key transcripts. We showed that VAChT-mutant mice had abnormal splicing of the *BACE1* gene, and that this was regulated by hnRNPA2/B1. This change in BACE1 splicing lead to an overall increase in protein levels of BACE1, altered APP processing and accumulation of soluble $A\beta_{1\text{-}42}$ in the brain of these animals. These pathological changes also involved age-related increased tau hyper-phosphorylation, and other neuronal abnormalities, ultimately leading to neuronal death in the hippocampus. These results indicate that alterations in RNA metabolism are a key mechanism by which cholinergic signalling in the brain can trigger Alzheimer's-like pathology in mice.

In chapter 6, we identified specific cellular mechanisms underpinning cholinergic regulation of hnRNPA2/B1. In both chapters 3 and 5 we provided evidence of the importance of this relationship to neuronal function. To study this relationship, we employed a series of genetically modified mouse models, pharmacology, and a

combination of *in vivo* and *in vitro* techniques. In VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ transgenic mice, we showed that reducing cholinergic signalling decreased protein levels of hnRNPA2/B1. Genetic manipulations that increased cholinergic signalling, by increasing gene copy number of VAChT, had the opposite effect, increasing hnRNPA2/B1 protein levels. We then provided biochemical evidence that regulation of hnRNPA2/B1 protein levels is not mediated by transcription, protein aggregation, or protein degradation. We found however that cholinergic signalling regulates the translation of hnRNPA2/B1. Furthermore, by combining in vitro and in vivo experiments, we demonstrated that M1 muscarinic receptors control hnRNPA2/B1 protein levels. In this chapter we outlined a sophisticated regulatory mechanism of hnRNPA2/B1 by cholinergic activity, complimenting findings of previous chapters.

7.2 Limitations and Future Studies.

The mouse line used for studies presented in Chapter 2, the ChAT-ChR2-EYFP mice were originally designed for optogenetic experiments. In these mice, the BAC construct was engineered to express an excitatory rhodopsin specifically in cholinergic neurons. However given the unique organization of the cholinergic gene locus (See Chapter 1.2.1), the VAChT gene is present in the BAC. Given our findings that these mice present several additional functional gene copies of VAChT, the utility of these mice for optogenetic experiments is questionable. Therefore future endeavours should focus on this question. A second mouse line was also generated using this same approach (Zhao et al., 2011), the B6.Cg-Tg(Chat-COP4*H134R/EYFP,Slc18a3)5Gfng/J mouse line. Evaluating whether or not this second mouse line also has additional copy numbers of VAChT would be critical. If these mice do not have a functional increase in VAChT it would be of importance to compare findings from optogenetic stimulation in the ChAT-ChR2-EYFP mice to evaluate the contribution of VAChT overexpression to these studies.

In chapter 3 of this thesis we used a genetic method to target the elimination of VAChT from the basal forebrain. This method is not without limitations and differential approaches should be used to confirm our findings. We have validated the use of stereotaxic injection of Cre viruses into specific populations of neurons to delete VAChT (Al-Onaizi et al., 2016). Delivering a Cre virus to the nucleus basalis magnocellularis (NBM) would provide a means to evaluate the specific contribution of cholinergic tone in the PFC, without manipulating acetylcholine release in other brain regions. Viral mediated elimination of VAChT to the NBM would also rule out potential developmental changes contributing to the phenotype of forebrain VAChT-deficient mice.

The major phenotype we identified for α7nAChR knockout mice in chapter 4 was impaired sustained attention. We were able to reverse this phenotype by activating β2nAChRs. A critical step would be to provide electrophysiological evidence that the drug effects occur in the PFC, as we only provided correlative evidence that the interaction between these receptors occurs in the PFC. One possibility is that the interplay between these receptors occurs at the same synapses in the PFC. Conversely, it is possible that the α7nAChR signalling and the β2nAChR signalling are separate and distinct. In agreement with the former possibility, α7nAChR (Duffy et al., 2009) and β2nAChR (Poorthuis et al., 2013) are found both pre and post-synaptically in the PFC. Genetically eliminating these receptors from specific synaptic locations would provide valuable insight into the exact interaction between these receptors and their relative contributions to attention.

In Chapter 5 of the thesis we used an *in silico* analysis of the data from our RNA-Sequencing experiment, to identify hnRNPA2/B1 as one of the RNA binding protein which mediates the alternative splicing in the hippocampus of forebrain VAChT-mutant mice. However hnRNPA2/B1 was not the sole RNA-binding protein identified by this analysis. Other candidates for potential splicing factors, identified in chapter 5 are RNA binding proteins that have also been implicated in neurodegenerative disorders such as CLEF4 (Gallo and Spickett, 2010), SRSF2 (Raj et al., 2014) and SART3 (Stamper et al., 2008). Similar *in vivo* and *in vitro* approaches used to study hnRNPA2/B1 in

chapters 5 and 6 could elucidate how cholinergic tone could regulate these proteins. Furthermore, the RNA binding protein analysis was limited to exon inclusion events, leaving an entire half of the data (the exon exclusion events) to be subjected to the same analysis. Analysing the exon exclusion event data will allow us to expand upon our findings and determine novel mechanisms by which cholinergic signalling can regulate RNA-metabolism.

The basis of the argument in chapter 5 is that VAChT deficiency leads to increased BACE1 activity, by mediating hnRNPA2/B1 protein levels. We proposed that this increase in BACE1 alters APP processing and increases soluble Aβ. It will therefore be critical to evaluate this hypothesis *in vivo*. To determine a causal role for BACE1 in the observed altered APP processing in the forebrain VAChT mutant mice, it would be important to inhibit BACE1 in vivo. This could be done either genetically or pharmacologically, and APP processing and soluble Aβ levels should then be assessed.

In chapter 6 we provided evidence that M1 muscarinic receptors regulate the translation of hnRNPA2/B1in hippocampal neurons. The M1 muscarinic receptor is a G_q coupled receptor and can signal through $G_{\alpha q}$ and $G_{\alpha s}$ (Thomas et al., 2008). This receptor can signal through various second messenger pathways including PKC signalling, PIP2 and modulation of intracellular calcium levels (Delmas and Brown, 2005). Given the potential signalling diversity of this receptor, determining the specificity of how M1 muscarinic receptors increases translation of hnRNPA2/B1 is critical to target this interaction for potential therapeutic benefits.

It would also be an important line of study to determine the time course of cholinergic regulation of hnRNPA2/B1. All *in vitro* studies presented in chapter 6 involved treating cultured neurons for 48 hours. Determining the shortest amount of time needed to change hnRNPA2/B1 protein levels would be critical for understanding the mechanism of regulation. These findings could then be validated *in vivo*. This could be achieved by pharmacological or chemogenetic based approaches, to stimulate M1 muscarinic pathway in mice.

7.3 Significance of Research and Conclusion.

Although the specific mechanisms by which cholinergic signalling in the brain can regulate cognition and neuronal function remain elusive, the data put forward in this thesis demonstrate the importance of cholinergic tone to both cognition and AD molecular pathology. A long line of literature has examined the role of cholinergic signalling in cognitive functioning. The data presented in this thesis advance this body of knowledge and expands upon how the different receptors for acetylcholine regulate different cognitive functions. In addition, we categorized for the first time the molecular hallmarks of long-term cholinergic dysfunction, using a wide range of approaches. In this thesis, I demonstrated a clear link between cholinergic dysfunction and the development of age-dependant molecular pathology in mice. Notably, I showed that some of these molecular changes brought on by long term cholinergic dysfunction are mediated by M1 muscarinic receptors. The data provided evidence that targeting this receptor may be beneficial to correct molecular alteration in patients with neurodegenerative disorders.

The current primary treatment for Alzheimer's disease in the clinic are acetylcholinesterase inhibitors. This line of treatment has shown improvements in cognition have been observed in patients, and are viewed as being moderately beneficial for patients (Courtney et al., 2004; Dubois et al., 2015; Kaduszkiewicz et al., 2005; Raina et al., 2008; Winblad et al., 2006). The data put forward in the thesis highlights key mechanisms by which cholinergic signalling in the brain can regulate cognition and molecular pathology. These mechanisms may serve as novel targets for the development of novel therapeutic interventions in humans affected by dementia. The discovery and development of positive allosteric modulators of cholinergic receptors (especially M1 muscarinic receptors) present an exciting new approach to the development of therapeutics for dementia, which could serve to target many of the mechanisms studied in this thesis.

References

Al-Onaizi, M.A., Parfitt, G.M., Kolisnyk, B., Law, C.S., Guzman, M.S., Barros, D.M., Leung, L.S., Prado, M.A., and Prado, V.F. (2016). Regulation of Cognitive Processing by Hippocampal Cholinergic Tone. Cereb Cortex.

Courtney, C., Farrell, D., Gray, R., Hills, R., Lynch, L., Sellwood, E., Edwards, S., Hardyman, W., Raftery, J., Crome, P.*, et al.* (2004). Long-term donepezil treatment in 565 patients with Alzheimer's disease (AD2000): randomised double-blind trial. Lancet 363, 2105-2115.

Delmas, P., and Brown, D.A. (2005). Pathways modulating neural KCNQ/M (Kv7) potassium channels. Nature reviews Neuroscience 6, 850-862.

Dubois, B., Chupin, M., Hampel, H., Lista, S., Cavedo, E., Croisile, B., Louis Tisserand, G., Touchon, J., Bonafe, A., Ousset, P.J.*, et al.* (2015). Donepezil decreases annual rate of hippocampal atrophy in suspected prodromal Alzheimer's disease. Alzheimer's & dementia : the journal of the Alzheimer's Association 11, 1041-1049.

Duffy, A.M., Zhou, P., Milner, T.A., and Pickel, V.M. (2009). Spatial and intracellular relationships between the alpha7 nicotinic acetylcholine receptor and the vesicular acetylcholine transporter in the prefrontal cortex of rat and mouse. Neuroscience 161, 1091-1103.

Gallo, J.M., and Spickett, C. (2010). The role of CELF proteins in neurological disorders. RNA biology 7, 474-479.

Kaduszkiewicz, H., Zimmermann, T., Beck-Bornholdt, H.P., and van den Bussche, H. (2005). Cholinesterase inhibitors for patients with Alzheimer's disease: systematic review of randomised clinical trials. BMJ 331, 321-327.

Poorthuis, R.B., Bloem, B., Schak, B., Wester, J., de Kock, C.P., and Mansvelder, H.D. (2013). Layer-specific modulation of the prefrontal cortex by nicotinic acetylcholine receptors. Cereb Cortex 23, 148-161.

Raina, P., Santaguida, P., Ismaila, A., Patterson, C., Cowan, D., Levine, M., Booker, L., and Oremus, M. (2008). Effectiveness of cholinesterase inhibitors and memantine for

treating dementia: evidence review for a clinical practice guideline. Annals of internal medicine 148, 379-397.

Raj, T., Ryan, K.J., Replogle, J.M., Chibnik, L.B., Rosenkrantz, L., Tang, A., Rothamel, K., Stranger, B.E., Bennett, D.A., Evans, D.A.*, et al.* (2014). CD33: increased inclusion of exon 2 implicates the Ig V-set domain in Alzheimer's disease susceptibility. Human molecular genetics 23, 2729-2736.

Stamper, C., Siegel, A., Liang, W.S., Pearson, J.V., Stephan, D.A., Shill, H., Connor, D., Caviness, J.N., Sabbagh, M., Beach, T.G.*, et al.* (2008). Neuronal gene expression correlates of Parkinson's disease with dementia. Movement disorders : official journal of the Movement Disorder Society 23, 1588-1595.

Thomas, R.L., Mistry, R., Langmead, C.J., Wood, M.D., and Challiss, R.A. (2008). G protein coupling and signaling pathway activation by m1 muscarinic acetylcholine receptor orthosteric and allosteric agonists. The Journal of pharmacology and experimental therapeutics 327, 365-374.

Winblad, B., Kilander, L., Eriksson, S., Minthon, L., Batsman, S., Wetterholm, A.L., Jansson-Blixt, C., and Haglund, A. (2006). Donepezil in patients with severe Alzheimer's disease: double-blind, parallel-group, placebo-controlled study. Lancet 367, 1057-1065.

Zhao, S., Ting, J.T., Atallah, H.E., Qiu, L., Tan, J., Gloss, B., Augustine, G.J., Deisseroth, K., Luo, M., Graybiel, A.M., and Feng, G. (2011). Cell type-specific channelrhodopsin-2 transgenic mice for optogenetic dissection of neural circuitry function. Nature methods 8, 745-752.

Appendix A

Regulation of cholinergic activity by the vesicular acetylcholine transporter

This is a copyedited, author-produced PDF of an article accepted for publication in *Biochemical Journal* following peer review.

Prado, V.F., Roy, A., Kolisnyk, B, Gros, R. and Marco, M.A. "Regulation of cholinergic activity by the vesicular acetylcholine transporter." Biochemical Journal 450, no. 2 (2013): 265-274.

Contributions to publication: BK assisted in the preparation of the review article

Biochem. J. (2013) **450**, 265–274 (Printed in Great Britain) doi:10.1042/BJ20121662

multimedia

REVIEW ARTICLE

Regulation of cholinergic activity by the vesicular acetylcholine transporter

Vania F. PRADO* [1], Ashbeel ROY* , Benjamin KOLISNYK*, Robert GROS* § and Marco A. M. PRADO* [1]

*[redacted]

Acetylcholine, the first chemical to be identified as a neurotransmitter, is packed in synaptic vesicles by the activity of VAChT (vesicular acetylcholine transporter). A decrease in VAChT expression has been reported in a number of diseases, and this has consequences for the amount of acetylcholine loaded in synaptic vesicles as well as for neurotransmitter release. Several genetically modified mice targeting the VAChT gene have been generated, providing novel models to understand how changes in VAChT affect transmitter release. A surprising finding is that most cholinergic neurons in the brain also can express a second type of vesicular neurotransmitter transporter that allows these neurons to secrete two distinct neurotransmitters. Thus a given neuron can use two neurotransmitters to regulate different physiological functions. In addition, recent data indicate that non-neuronal cells can also express the machinery used to synthesize and release acetylcholine. Some of these cells rely on VAChT to secrete acetylcholine with potential physiological consequences in the periphery. Hence novel functions for the oldest neurotransmitter known are emerging with the potential to provide new targets for the treatment of several pathological conditions.

Key words: Alzheimer's disease, heart failure, Parkinson's disease, sepsis, synaptic vesicle, vascular dementia.

INTRODUCTION

Cholinergic neurons in the CNS (central nervous system) and in the periphery secrete the neurotransmitter ACh (acetylcholine) to regulate a plethora of physiological functions. In addition to ACh, many cholinergic neurons in the brain can also secrete the neurotransmitter glutamate [1], whereas cholinergic neurons in the periphery can also secrete a number of peptides and ATP, suggesting the potential for sophisticated modulation of physiological functions by these neurons. Moreover, ACh is also present in a number of non-neuronal cells, where it may have paracrine or autocrine functions [2,3]. Given its cationic nature, ACh does not diffuse effectively through membranes; therefore, a transport mechanism is required for this neurotransmitter to be secreted. Although certain organic cation transporters can carry ACh [2], both in neurons, as well as certain non-neuronal tissues, this chemical messenger is first stored in vesicles prior to being released by exocytosis [4–6].

ACh synthesis (Figure 1) depends on the uptake of the ACh precursor choline by CHT1 [high-affinity choline transporter/SLC5A7 (solute carrier family 5 member 7)] that is mainly expressed in cholinergic neurons [7], although it can also be found in certain non-neuronal cells [2,5]. In the cytoplasm of nerve endings, ACh is synthesized by the enzyme ChAT (choline acetyltransferase) (EC 2.3.1.6) and is then loaded into synaptic vesicles by VAChT [vesicular ACh transporter/SLC18A3 (solute carrier family 18 member 3)] [4,8]. VAChT is a 12 transmembrane domain protein that is part of a Major Facilitator Superfamily of transporters [9]. This superfamily also includes the neurotransmitter transporters VMAT (vesicular monoamine transporter) 1 and VMAT2, which share a high degree of homology with VAChT in their transmembrane segments. These transporters use the electrochemical gradient generated by a V-type proton ATPase to transport and accumulate neurotransmitters in vesicles [8].

Synaptic vesicles accumulate thousands of ACh molecules to form a quantum. Interestingly, *in vitro* analysis suggests that VAChT is a very slow transporter [10], thus serving as a limiting factor in the recycling of functional cholinergic synaptic vesicles (loaded with ACh) to maintain neurotransmitter release. Indeed, recent experiments in neurons using glutamate uncaging indicate that the VGLUT (vesicular glutamate transporter) is also a very slow transporter [11]. Therefore expression of vesicular transporters and their activity may have major influences on the release of ACh. In the present review we will evaluate novel genetic insights regarding the role of VAChT for transmitter release in neuronal and non-neuronal cells as well as the functional consequences of alterations in VAChT expression. Excellent reviews on the use of genetically modified mice to probe for the specific roles of subtypes of muscarinic and nicotinic receptors have been published previously [12–18], so we will not discuss these results.

VAChT BIOCHEMISTRY AND CELL BIOLOGY

The structure of VAChT has not been resolved experimentally, but a three-dimensional model for this transporter has been proposed based on structural information from two members of the Major Facilitator Superfamily (lactose permease and glycerol 3-phosphate) [9]. The 12 transmembrane domains of VAChT are proposed to fold into two main bundles comprising

Abbreviations used: ACh, acetylcholine; ChAT, choline acetyltransferase; CHT1, high-affinity choline transporter 1; CNS, central nervous system; DA, dopamine; GABA, γ-aminobutyric acid; GI, gastrointestinal; hnRNP, heterogeneous nuclear ribonucleoprotein; IL, interleukin; KD, knockdown; KD^{HET}, heterozygous KD; KD^{HOM}, homozygous KD; KO, knockout; LDCV, large-dense core vesicle; LTP, long-term potentiation; MEPP, miniature-endplate potential; MSN, medium spiny neuron; MWM, Morris water maze; NMJ, neuromuscular junction; OCT, organic cation transporter; PKC, protein kinase C; VAChT, vesicular ACh transporter; VGLUT, vesicular glutamate transporter; VMAT, vesicular monoamine transporter.

[1] Correspondence may be addressed to either of these authors (email [redacted] or [redacted]).

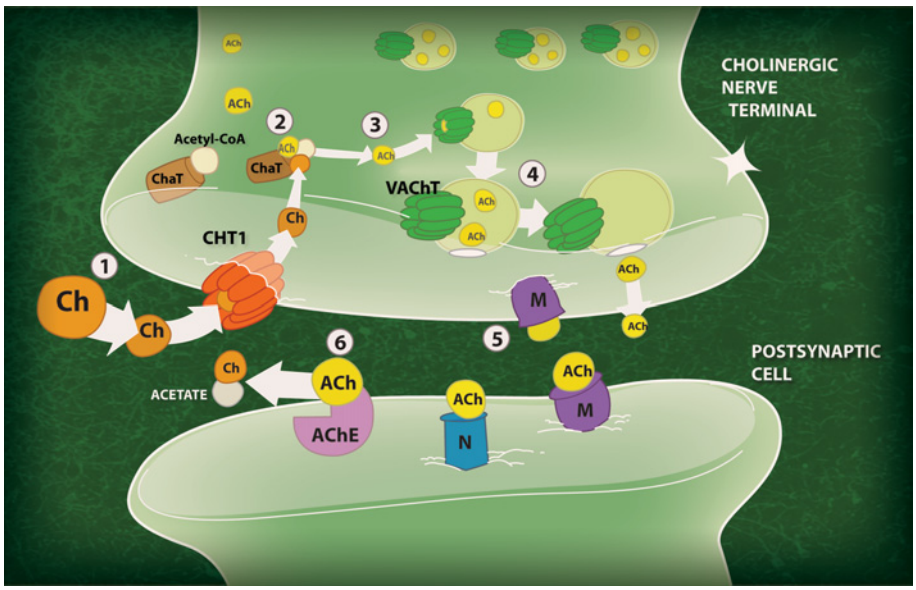


Figure 1 Schematic drawing of ACh storage and release

(**1**) Uptake of the ACh precursor choline by CHT1 that is mainly expressed in cholinergic neurons. (**2**) In the cytoplasm of nerve endings, ACh is synthesized by the enzyme ChAT, and then it is loaded into synaptic vesicles (**3**) by VAChT. (**4**) Upon arrival of the nerve impulse, vesicles fuse to the plasma membrane and release the neurotransmitter that can then signal through nicotinic (N) and muscarinic (M) receptors (**5**). ACh is rapidly degraded into acetate and choline (**6**) by the enzyme AChE (acetylcholinesterase). The number of transmembrane domains for CHT1 is 13 [160] and for VAChT is 12. These are not shown faithfully in the Figure due to space limitations. Ch, choline.

transmembrane helices I–VI and VII–XII respectively [19], with N- and C-terminal regions directed to the cytoplasm. According to this model, a central transport path is formed by these two bundles and a rocker motion of the bundles allows for exposure of the substrate-binding site to the cytoplasm or to the interior of the synaptic vesicle. VAChT exchanges two luminal protons for each cytoplasmic ACh molecule [20]. Site-directed mutagenesis studies suggest that the ACh-binding site is located close to Trp^{331} at the beginning of transmembrane helix VIII, in the luminal part of the transport channel [21]. Asp^{398} is suggested to be involved in translocation of one of the protons [22,23].

Studies in PC12 cells overexpressing human VAChT [10] indicate that transport of [^{3}H]ACh by VAChT is saturable, with an apparent K_m value of 1 mM and a V_{max} value of 580 pmol/min/mg. *In vivo*, VAChT concentrates ACh inside synaptic vesicles by 100-fold. This gradient is around 30-fold smaller than that predicted from the available free energy from the exchange of two protons [8], suggesting that ACh storage is regulated [4,24]. Although the mechanisms for regulation are unknown, it has been demonstrated that the amount of ACh stored per vesicle depends on the amount of VAChT that is expressed [25–27]. Thus VAChT is likely to be rate limiting for ACh release. Indeed, early pharmacological experiments using the drug vesamicol [(−)-*trans*-2-(4-phenylpiperidino) cyclohexanol], the prototype VAChT inhibitor, provided evidence that ACh storage in synaptic vesicles is critical for release; although considerations of pharmacological specificity *in vivo* need to be considered (for a review see [24]). For example, early work used high concentrations of vesamicol and binding to other unrelated targets was observed in the peripheral nervous system [28]. Also, interaction with sigma receptors in the brain has been described. However, novel vesamicol analogues have been produced showing higher specificity for VAChT [29,30].

In the striatum, cholinergic neurons were shown to express VGLUT3, and this transporter activity influences ACh loading in synaptic vesicles by a process named vesicular synergy [31]. The exact mechanism is not yet clear, but it is likely that negatively charged glutamate may affect the ΔpH value to increase transport activity [1,32]. Hence, in addition to accumulating glutamate inside cholinergic vesicles, VGLUT3 may also influence ACh storage. Dopaminergic neurons, on the other hand, express VGLUT2, and DA (dopamine)/glutamate co-transmission has been suggested to play important roles during development and to regulate DA-dependent functions [33,34]. Interestingly, recent experiments suggest that the closely related transporter, VMAT2, mediates release of GABA (γ-aminobutyric acid) in the striatum [35]. This adds to the remarkable lack of specificity for these transporters. Indeed, choline can also be taken up by VAChT, although the affinity for ACh is 7-fold higher than that of choline [36,37].

Vesamicol, a tertiary amine that spontaneously passes through membranes, binds to VAChT and inhibits the transport of ACh [8,22,23,38]. Vesamicol is a non-competitive inhibitor of ACh transport, exhibiting a dissociation constant of 20 nM. Phosphorylation of VAChT at a PKC (protein kinase C) site in the C-terminal domain of VAChT blocks inhibition of transport by vesamicol and a high-affinity analogue of vesamicol [39–41]. However, it has not been determined whether VAChT phosphorylation is important for the modulation of ACh storage *in vivo*. Vesamicol and related compounds bind to VAChT with high affinity and readily cross the blood–brain barrier, therefore intensive efforts have been dedicated to develop analogues of vesamicol that emit either a positron or a gamma photon, suitable for imaging by PET (positron emission tomography) or SPECT (single-photon emission computed tomography) respectively [29,30,42–44]. These compounds have potential applications in the diagnosis of a number of diseases characterized by cholinergic dysfunction including Alzheimer's disease, Down's syndrome, Parkinson's disease, autonomic dysfunction in cardiovascular diseases and schizophrenia.

VAChT LOCALIZATION AND TRAFFICKING

The molecular basis for localization of vesicular transporters in synaptic vesicles has been reviewed elsewhere [45,46]. VAChT is found predominantly in synaptic vesicles in nerve terminals [47]. Early work on the trafficking of VMATs and VAChT indicated that, in cultured cells, these transporters differ in their localization; VAChT was predominantly present in synaptic vesicles, whereas VMATs were found mostly in LDCVs (large-dense core vesicles) [48-50]. Synaptic vesicles and LDCVs are present in most neurons and may regulate the secretion of classical neurotransmitters and peptides or other neuromodulators respectively. Additional studies demonstrated that the C-terminal region of VAChT contains a di-leucine motif required for clathrin-mediated endocytosis [51–54]. Interestingly, this di-leucine motif is regulated by a phosphorylation site that can alter the proportion of VAChT present in small synaptic vesicles or LDCVs [49], suggesting that ACh storage in distinct types of vesicles might occur and be subject to regulation. The C-terminal region of VAChT is also important for its localization in small synaptic vesicles [50,53,55]. Extensive mutational analysis failed to uncover other motifs in the C-terminal tail, other than the di-leucine motif, that can influence VAChT trafficking [53,55]. Interestingly, endocytosis of CHT1, which is also located in synaptic vesicles, is dependent on clathrin and on a di-leucine-like motif present in its C-terminal tail [7,56–58].

A small number of proteins have been described to interact with VAChT. Notably, clathrin adaptor proteins interact with the C-terminal tail of VAChT [52]. In addition, a functional interaction between VAChT and synaptobrevin has also been described in *Caenorhabditis elegans* [59]. These interactions are believed to participate in VAChT trafficking. SEC14, a phosphatidylinositol transfer protein, has also been shown to interact with VAChT [60]; however, the functional consequences of this interaction are unknown.

VAChT expression levels have been shown to change in response to drug treatments [61], as well as in diseases including Alzheimer's disease [62,63], sepsis [64], hypertension [65] and Huntington's disease [66]. Small changes in the expression of VAChT in vesicles may have the potential to change synaptic transmission, as the amount of transmitter released by a single vesicle does not seem to be enough to saturate post-synaptic receptors [67]. This appears to be the case at the neuromuscular junction, as suggested by the extensive variability of quantum size [24]. In central cholinergic terminals, especially where cholinergic terminals do not have opposed post-synaptic cells forming classical synapses, volume transmission may not be enough to saturate ACh receptors [68]. However, classical forms of neurotransmission may also be relevant for ACh in the brain. For example, VAChT-expressing terminals are found close to $\alpha 7$ nicotinic ACh receptors thus suggesting that classical synapses are involved in fast transmission of information by ACh in the brain, in addition to volume transmission [69].

REGULATION OF ACh RELEASE BY VAChT

The relationship between ACh storage and release is complex [24]. Experiments using vesamicol and vesamicol analogues have demonstrated that inhibition of ACh transport into synaptic vesicles decreases ACh release from nerve terminals [8,24]. Overexpression of VAChT in immature neurons [25] has also been used to investigate the *in vitro* relationship between ACh storage and release. These elegant experiments demonstrated that increased VAChT expression augmented the amplitude (quantal size) and frequency of miniature excitatory currents, presumably by increasing the number of vesicles capable of storing ACh [25]. In addition, mice with increased VAChT expression show increased ACh release [70]. Other experiments demonstrated that VAChT activity is required for physiological storage of ACh, as VAChT-KO (knockout) mice do not survive following birth owing to compromised respiratory activity [71]. Decreased levels of VAChT affect both the peripheral nervous system and the CNS [26,27,71–76], suggesting that, in contrast with the VMAT family which has two members [77], VAChT is the unique transporter for ACh. Neuromuscular phenotypes in VAChT-KO mice were similar, if not identical, to phenotypes described for ChAT-KO mice [78,79]. The normal apoptotic process that prunes the number of motoneurons during development is compromised by lack of VAChT and motoneuron numbers are increased. At the NMJ (neuromuscular junction), nerve endings show increased size and number. Moreover, skeletal muscle presents degeneration and atrophy indicating that, during development, secretion of ACh required for neuromuscular development depends mainly on VAChT activity. Remarkably, the levels of ACh in terminals lacking VAChT were increased, suggesting that feedback inhibition of ACh synthesis is not operational in cholinergic nerve terminals [71].

Surprisingly, electrophysiological analysis of VAChT-KO mice detected small-amplitude MEPPs (miniature-endplate potentials) of very low frequency in the NMJ preparations of null embryos, suggesting that some diffusion and accumulation of ACh in synaptic vesicles occurs in the absence of VAChT. However, this process is insufficient to sustain minimal levels of ACh release at the NMJ. In agreement with these data, release of newly synthesized ACh from brain synaptosomes is completely abolished in VAChT-KO mice [71].

Interestingly, a 50 % decrease in VAChT expression in heterozygous VAChT-KO mice did not affect muscular function [72]. Conversely, these mutant mice present behavioural deficits in object recognition memory [72,80]. These results suggest that central cholinergic synapses are much more dependent on VAChT than NMJ terminals to sustain neurotransmitter release. This is probably because of reduced numbers of synaptic vesicles, or their frequent reuse, in central nerve terminals compared with the NMJ. Further insight into the mechanisms through which altered VAChT expression regulates ACh release came from studies using VAChT-KD (knockdown) mice [26,27]. VAChT-KD^{HET} (heterozygous KD) mice show a 40 % decrease in VAChT expression. Similar to heterozygous VAChT-KO mice, VAChT-KD^{HET} mice do not present muscular dysfunction. Microdialysis analysis in freely moving mice showed that VAChT-KD^{HET} mice have decreased levels of ACh release in the brain. These mutants show deficits in object recognition memory and social recognition memory [26]. These results suggest that the NMJ has a much higher safety factor than central synapses to maintain ACh release. VAChT-KD^{HOM} (homozygous KD) mice have a 70 % decrease in VAChT expression and provide a model for understanding the consequences of profound decrease in VAChT expression for ACh release. Quantal analysis demonstrated that VAChT-KD^{HET} mice have normal MEPP frequency and slightly reduced MEPP amplitude, indicative of the amount of ACh within vesicles. In contrast, VAChT-KD^{HOM} mice show reduced amplitude of MEPPs, consistent with decreased ACh storage, but also a 50 % decrease in the frequency of MEPPs [26,27]. As a consequence of these molecular changes, VAChT-KD^{HOM} mice are myasthenic. Moreover, endplate potentials were also reduced in these mutants [27]. Independent analysis of exocytosis and endocytosis using the fluorescent dye FM1-43 indicated that synaptic vesicle fusion and recycling were not affected in these mutant mice. Furthermore, post-tetanic potentiation is compromised in the NMJ of these

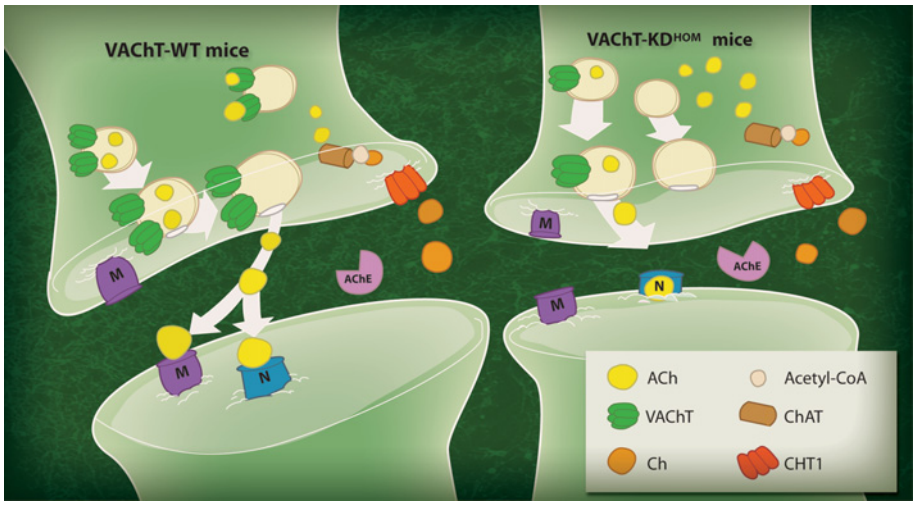


Figure 2 Cholinergic nerve terminal in mice with decreased VAChT expression

The left-hand side shows a nerve terminal in wild-type (WT) mice. The right-hand side shows a nerve terminal in VAChT-KD^{HOM} mice. Some synaptic vesicles will have no transporter and will be unable to store significant levels of ACh. These vesicles will compete for release sites and decrease fusion of vesicles fully loaded with ACh. An animated version of this Figure can be found at http://www.biochemj.org/bj/450/0265/bj4500265add.htm. Ch, choline; M, muscarinic receptor; N, nicotinic receptor.

VAChT mutant mice, suggesting that ACh storage can regulate synaptic plasticity [27]. Together, these experiments suggest that reduced levels of VAChT affect ACh storage in synaptic vesicles (Figure 2).

The number of copies of VAChT in a synaptic vesicle is unknown. Studies considering other vesicular transporters estimated that one to three transporters are present in one synaptic vesicle [24]. However, central synapses may contain up to ten copies of neurotransmitter transporters [81]. It is tempting to hypothesize that, in conditions of reduced VAChT expression, some synaptic vesicles will have no transporter and will be unable to store significant levels of ACh (Figure 2). These vesicles would not be able to sustain ACh release; however, because exocytosis and endocytosis in these ACh-empty vesicles is normal, they would compete for releasing sites at the NMJ. The net effect would be a reduction in the frequency of fusion of vesicles containing ACh, which can be detected using electrophysiology (MEPP frequency). These results suggest that even small changes in the levels of VAChT found in Alzheimer's disease and other pathological conditions would have drastic effects on ACh release in the brain, with a reduction in the amount of transmitter released by vesicles, but also a decrease in the number of synaptic vesicles capable of sustaining ACh release.

VAChT AND CHOLINERGIC TONE IN THE CNS

Cholinergic tone has been proposed to modulate a number of brain functions including learning, memory, attention, arousal, sleep, food intake and drug abuse [82–88]. Owing to space limitations we will not discuss these effects in the present review; rather, we will focus on novel aspects of cholinergic neurotransmission uncovered in recent years.

It has been shown that a large number of cholinergic neurons in the CNS co-express a member of the vesicular glutamate-transport protein family and therefore have the potential to co-release glutamate. To note, cholinergic neurons in the habenula co-express VGLUT1 [89], whereas basal forebrain cholinergic neurons projecting to the amygdala [90] as well as tonically active cholinergic interneurons in the striatum co-express VGLUT3 [91]. In the retina, GABA has been shown to be co-released with ACh [92]. In cultured cholinergic neurons from the basal forebrain, glutamate release has been shown to occur [90]. More recently, evidence from optogenetic studies indicates that cholinergic neurons can co-release glutamate in brain slices [89,93]. Brief photostimulation of cholinergic axonal terminals was shown to induce fast excitatory post-synaptic currents mediated by glutamate receptors [89,93], whereas tetanic photostimulation generated slow post-synaptic currents mediated by nicotinic receptors in the habenula [89].

Genetic approaches have been used to investigate the physiological significance of ACh/glutamate co-transmission in the striatum (Figure 3). Studies using mice with null expression of VGLUT3 (VGLUT3-KO) show evidence that VGLUT3 is co-expressed with VAChT in synaptic vesicles and facilitates ACh filling of these vesicles [31]. VGLUT3-KO mice are hyperactive, more responsive to cocaine and less prone to haloperidol-induced catalepsy than their wild-type littermates [31]. Given that mice with ablated cholinergic neurons in the striatum show a similar phenotype [94,95], it was initially suggested that these behavioural changes resulted from the decreased striatal ACh release observed in VGLUT3-KO mice [31]. Recently it was demonstrated that mice with selective elimination of VAChT from striatal cholinergic interneurons (VAChT$^{D2-Cre-flox/flox}$ mice) are not hyperactive and show minimal alteration in behavioural responses to cocaine. These results strongly suggest that glutamate released from cholinergic neurons, rather than ACh, is critical for cocaine-induced behavioural manifestations [76]. Conversely, it was shown that elimination of striatal ACh release affects DA metabolism. It also appeared to affect the response of MSNs (medium spiny neurons) to DA where up-regulation of DA receptors and a change in behavioural responses to dopaminergic agonists was observed [76]. These data indicate that cholinergic interneurons use two distinct neurotransmitters to differentially regulate behaviour. Moreover, synchronized activity of cholinergic interneurons was shown to depolarize DA nerve terminals directly and evoke DA release, independently of the action potentials in DA soma [96,97], indicating that the

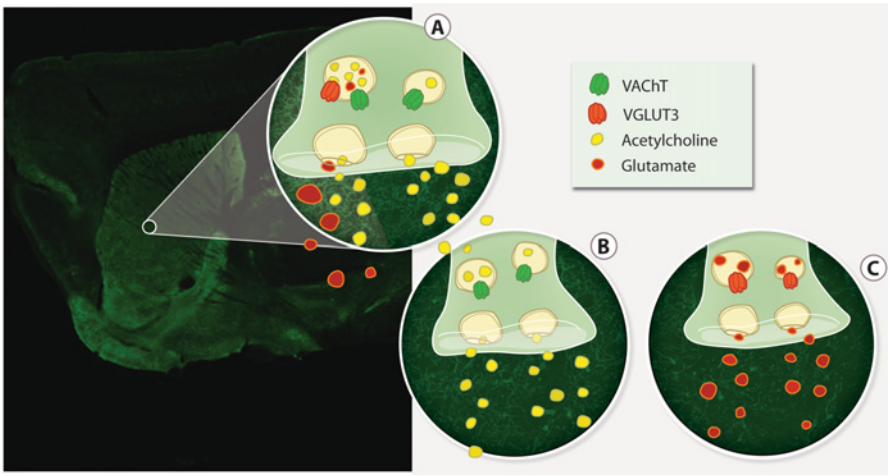


Figure 3 ACh/glutamate co-transmission in the mammalian striatum, and the effects of molecular manipulations

(**A**) Schematic representation of the normal ACh/glutamate co-transmission and vesicular synergy in the striatum. (**B**) Loss of VGLUT3 results in elimination of glutamate release. (**C**) Targeted KO of VAChT in the striatum results in loss of ACh release.

ACh-driven DA signal has crucial outcomes for DA nerve function. Striatal cholinergic neurons can also regulate MSNs by activating GABA release from interneurons and silencing MSNs [98]. Importantly, these findings demonstrate that co-transmission has the potential to significantly affect the functioning of striatal neurons and hence of basal ganglia. Additionally, these results suggest that other roles previously attributed to ACh in striatal function might either result from glutamate released from cholinergic interneurons or depend on the combined action of both transmitters.

Basal forebrain cholinergic neurons, which provide the major input to the cortex and hippocampus, undergo moderate degenerative changes during normal aging, and the resulting cholinergic hypofunction has been associated with age-related memory deficits [99]. A more profound alteration in basal forebrain cholinergic cells is thought to underlie some of the cognitive and behavioural symptoms observed in both Alzheimer's disease and vascular dementia as well as in Parkinson's disease [100–102]. Importantly, VAChT levels are decreased in the brains of Alzheimer's disease patients [62,63].

Studies using animal models of cholinergic dysfunction generated by ablation of basal forebrain cholinergic neurons using electrolytic or excitotoxic methods, as well by the more selective strategy of cholinergic immunolesion, have given inconsistent results concerning the cognitive and behavioural processes that are affected by altering cholinergic transmission [103]. These studies have been hampered by the fact that both non-cholinergic and cholinergic projection neurons are destroyed in many cases, or that the lesions produced do not fully deplete cholinergic neurons. Moreover, these studies cannot separate potential roles of ACh and glutamate that have the potential to be secreted together by these neurons. Genetically modified mice with selective elimination of VAChT expression in forebrain cholinergic neurons provide a model to isolate the consequences of cholinergic deficiency in dementia [75]. Behavioural analysis of mice with selective forebrain VAChT deficiency (VAChT$^{Six3-Cre-flox/flox}$ mice) indicates that these mice are hyperactive [75]. Other mouse lines with decreased cholinergic tone also show augmented locomotion [74]. Hyperactivity in mice is related to a number of possible psychiatric-like behaviours, including a potential increase in anxiety-like behaviour, but this is not the case in these mutant mice. Importantly, the hyperactivity observed in VAChT$^{Six3-Cre-flox/flox}$ mice is not due to elimination of VAChT in the striatum [76]. Thus these data highlight the importance of basal forebrain cholinergic neurotransmission for the modulation of locomotor activity. Additionally, forebrain VAChT deficiency compromised the ability of mice to use spatial cues to find the platform in the MWM (Morris water maze) [75]. Although these mice show deficits on MWM acquisition, they develop alternative strategies to recall the correct location of the platform in probe trials. Interestingly, forebrain VAChT deficiency impaired the capacity of mice to extinguish a previous location and learn a new platform location, suggesting that ACh is important for behavioural flexibility. Accompanying this spatial memory impairment, we found an impairment in LTP (long-term potentiation) in VAChT$^{Six3-Cre-flox/flox}$ mice. This effect on LTP did not involve changes in glutamatergic synaptic transmission, as input/output relationship and paired-pulse facilitation were not affected [75]. Together, these results suggest that VAChT$^{Six3-Cre-flox/flox}$ mice have cellular and behavioural deficits that prevent proper encoding of spatial memory information, which is one of the first behavioural deficits observed in Alzheimer's disease patients. Hence, these mice may provide a more reliable model of neurochemical changes in Alzheimer's disease and other types of dementia. Potential long-term functions affected by cholinergic deficiency are still largely unknown. Exciting new data suggest that loss of the nuclear ribonucleoproteins hnRNP (heterogeneous nuclear ribonucleoprotein) A/B family, which is observed in the entorhinal cortex of Alzheimer's disease patients, is induced by cholinergic deficiency [104]. Furthermore, loss of the hnRNP splicing factors was shown to cause alternative splicing impairments, dendrite loss in primary neurons and cognitive impairments [104].

REGULATION OF CHOLINERGIC TONE IN THE AUTONOMIC NERVOUS SYSTEM BY VAChT

ACh is the primary chemical neurotransmitter at parasympathetic nerve endings and modulates the function of many peripheral organs in the body. VAChT-positive neurons have been identified in several different organs including the retina, GI (gastrointestinal) tract and respiratory tract, as well as the heart [105].

In the heart, co-ordinated interplay between the two branches of the ANS (autonomic nervous system) is important in maintaining proper function. Activation of the sympathetic branch increases heart rate and contractile force, whereas activation of the parasympathetic branch reduces heart rate by altering the conduction velocity of both the sinoatrial and atrioventricular nodes [106]. It has long been recognized that overactivation of the sympathetic tone contributes to cardiac dysfunction [107–109]; in contrast, much less is known about the role of failing cholinergic neurotransmission in cardiac disease. Vagal stimulation has been shown to improve the outcomes in experimental heart failure [110–118] and it is an approach currently being explored to ameliorate a number of diseases in humans. Recent studies using VAChT-KD^{HOM} mice, which show a systemic reduction in VAChT, provided direct evidence that decreased cholinergic neurotransmission also causes plastic alterations that contribute to heart dysfunction [73,119]. The hearts of VAChT-KD^{HOM} mice have altered calcium handling, show changes in myocyte contractility and express several markers of cardiac stress, which are activated during cardiac remodelling and heart failure [73]. Importantly, all of these changes can be reversed through treatment with pyridostigmine, a peripheral cholinesterase inhibitor, suggesting that cardiac dysfunction in these mice results from a deficiency in cholinergic tone [73]. Mice lacking M_2 muscarinic receptors show increased cardiac stress [120], and experiments using mice lacking one of the high-affinity choline transporter alleles confirmed that decreased cholinergic tone affects heart function [121].

The cholinergic system is important in regulating the innate immune response. In fact, several studies have provided novel insight into the specific mechanisms through which neuronal ACh can act peripherally to control the immune response. This inflammatory reflex, termed the cholinergic anti-inflammatory pathway, is dependent on the peripheral actions of ACh released from the vagus nerve [110,122,123]. ACh binds to α7 nAChRs (nicotinic acetylcholine receptors) on macrophages and thereby inhibits the release of pro-inflammatory cytokines including TNFα (tumour necrosis factor α), IL (interleukin)-1β and IL-6 [122,123]. Inhibition of cytokine release has been shown to be beneficial in several disease states, including endotoxaemia, sepsis and heart failure [124–127]. Consistent with these results, VAChT-KD^{HOM} mice have been shown to develop an increased inflammatory immune response when infected with parasites [128], indicating disturbance of the cholinergic anti-inflammatory reflex in this mutant.

In the retina, ACh is released in response to light stimulation and leads to direct and rapid excitation of the retinal ganglion cells [129]. Release of ACh in the enteric nervous system induces smooth muscle contractions in the GI tract [130,131]. Cholinergic signalling also leads to smooth muscle contraction in the respiratory tract [132]. Whether decreased cholinergic tone has pathological implications related to eye function, the enteric system or the respiratory tract has not yet been systematically investigated.

NON-NEURONAL CHOLINERGIC MACHINERY

ACh is also produced in many different cell types, leading to the idea that a non-neuronal cholinergic system plays a significant role in regulating various physiological functions [133]. Indeed, the machinery necessary to produce ACh as well as the neurotransmitter itself has been identified in a range of cells including lymphocytes [134,135], epithelial cells [136–138], vascular endothelial cells [139] and the α-cells of the pancreas [6]. Furthermore, this machinery has been identified in cardiomyocytes [5,140,141].

Immune cells, including lymphocytes, possess the machinery necessary to synthesize ACh [134,135,142,143]. It has been recently demonstrated that a small population of T-lymphocytes can synthesize and release ACh in response to autonomic nervous system activity in the spleen as part of the cholinergic anti-inflammatory pathway [144]. It is important to note that T-cells as well as macrophages express both muscarinic and nicotinic ACh receptors and are also able to produce ACh [145]. The exact mechanism by which ACh is released from these cells to regulate the immune system has not yet been uncovered.

The ability of epithelial cells to synthesize ACh is well characterized; in fact, it has previously been reported that this molecule can be secreted from cultured bronchial epithelial cells [137]. However, ACh release from epithelial cells does not appear to be dependent on VAChT, but rather on the organic cation transporters OCT (organic cation transporter) 1 and OCT2 [146,147]. ACh released by epithelial cells acts through both nicotinic as well as M_1 muscarinic receptors and increases their proliferation rate [148]. Moreover, in a number of cancer cells, particularly in lung cancer, ACh has been shown to play an autocrine role [149–151].

ACh is important in regulating insulin release from β-cells of the pancreas. It has long been suggested that the source of this ACh is the parasympathetic nerve endings in the endocrine pancreas, which can trigger insulin release following binding to the M_3 muscarinic receptor [152,153]. Interestingly, recent work has revealed that, although insulin secretion from β-cells is regulated by neuronal parasympathetic signalling in mice, humans possess the machinery to synthesize and release ACh from α-cells. VAChT plays an important role in the release of ACh from α-cells as positive immunostaining was observed for the transporter in these cells and pharmacological manipulation blocked ACh release. This suggests that ACh is maintained in exocytotic vesicles within α-cells and is released quantally in a manner similar to that observed in neurons [6]. Owing to the fact that β-cells are mostly localized close to α-cells in the pancreatic islets, the non-neuronal ACh released by α-cells can act as a paracrine molecule on neighbouring β-cells and initiate the insulin secretion cascade [6].

Vascular endothelial cells play a critical role in the regulation of blood pressure by inducing the relaxation of vascular smooth muscle cells. This pathway is well characterized and ACh is known to mediate vasodilation through binding to muscarinic ACh receptors on endothelial cells. This interaction leads to the production of the EDRF (endothelium-derived relaxing factor), now known to be nitric oxide, which leads to vasodilation [154]. Although ACh appears to have a profound effect on vascular function, it is important to note that parasympathetic innervation of endothelial cells is virtually non-existent and there are high levels of acetylcholinesterases in circulation. It is now evident that endothelial cells have the ability to synthesize and secrete ACh via a PKC-independent mechanism [139,155]. Importantly, previous work has confirmed the presence of VAChT in two different endothelial cell culture models, suggesting that ACh may be released from these cells via a VAChT-dependent mechanism [156,157].

It has previously been proposed that cardiomyocytes are able to synthesize and release ACh at the cellular level as they possess the machinery for production of ACh [140,141]. Owing to limited innervation of ventricular cardiomyocytes by parasympathetic neurons [158,159], it has been suggested that ventricular cardiomyocytes may synthesize ACh. VAChT was shown to be present in vesicles in cardiomyocytes [141]

suggesting a quantal release of ACh from these cells [5]. In cell culture models, isoproterenol and other adrenergic activators can induce cellular hypertrophy and remodelling. ACh released by cardiomyocytes plays an important role in protecting these cells against isoproterenol-induced cardiomyocyte remodelling [5]. Furthermore, expression of both VAChT and ChAT was increased in cultured cardiomyocytes treated with adrenergic drugs [5], suggesting a potential mechanism for regulation of this machinery. This non-neuronal ACh may then act in an autocrine/paracrine fashion to enhance neuronal cholinergic signalling. Although demonstrated only *in vitro*, these results suggest a novel mechanism to protect the heart under stressful conditions. These data provide for an unanticipated mechanism by which non-neuronal ACh, secreted from VAChT-positive vesicles, may play an important role in cardiac function.

CONCLUSION

The potential to uncover novel physiological functions of ACh using genetically modified mice, in which the cholinergic machinery can be spatially and temporally targeted, has already changed our understanding of functions by this neurotransmitter. Otto Loewi's [161] findings of ACh as a neurotransmitter can now be expanded to a role in paracrine/autocrine communication in a number of non-neuronal cells. By using the Cre/*lox* system, BAC (bacterial artificial chromosomes) transgenic mice and optogenetics, we now have the ability to activate or inactivate the cholinergic machinery or cholinergic neurons at will. These novel approaches will lead to increased knowledge of how ACh contributes to different bodily functions. Additionally, these approaches will be fundamental to unravelling how different populations of cholinergic neurons in the brain can regulate distinct biochemical and physiological processes. It is tempting to speculate that optogenetics, used to activate or inactivate cholinergic neurons in the peripheral nervous system, may be effectively used in the future as treatment for a number of pathologies involving dysregulated sympathetic or parasympathetic activity. Finally, inactivation of cholinergic machinery in non-neuronal tissues using genetically modified mice will provide the ultimate proof for the physiological significance of non-neuronal release of ACh.

REFERENCES

1 El Mestikawy, S., Wallen-Mackenzie, A., Fortin, G. M., Descarries, L. and Trudeau, L. E. (2011) From glutamate co-release to vesicular synergy: vesicular glutamate transporters. Nat. Rev. Neurosci. **12**, 204–216
2 Wessler, I. and Kirkpatrick, C. J. (2008) Acetylcholine beyond neurons: the non-neuronal cholinergic system in humans. Br. J. Pharmacol. **154**, 1558–1571
3 Wessler, I. K. and Kirkpatrick, C. J. (2012) Activation of muscarinic receptors by non-neuronal acetylcholine. Handb. Exp. Pharmacol. **208**, 469–491
4 Prado, M. A., Reis, R. A., Prado, V. F., de Mello, M. C., Gomez, M. V. and de Mello, F. G. (2002) Regulation of acetylcholine synthesis and storage. Neurochem. Int. **41**, 291–299
5 Rocha-Resende, C., Roy, A., Resende, R., Ladeira, M. S., Lara, A., de Morais Gomes, E. R., Prado, V. F., Gros, R., Guatimosim, C., Prado, M. A. and Guatimosim, S. (2012) Non-neuronal cholinergic machinery present in cardiomyocytes offsets hypertrophic signals. J. Mol. Cell. Cardiol. **53**, 206–216
6 Rodriguez-Diaz, R., Dando, R., Jacques-Silva, M. C., Fachado, A., Molina, J., Abdulreda, M. H., Ricordi, C., Roper, S. D., Berggren, P. O. and Caicedo, A. (2011) Alpha cells secrete acetylcholine as a non-neuronal paracrine signal priming beta cell function in humans. Nat. Med. **17**, 888–892
7 Ribeiro, F. M., Black, S. A., Prado, V. F., Rylett, R. J., Ferguson, S. S. and Prado, M. A. (2006) The 'ins' and 'outs' of the high-affinity choline transporter CHT1. J. Neurochem. **97**, 1–12
8 Parsons, S. M. (2000) Transport mechanisms in acetylcholine and monoamine storage. FASEB J. **14**, 2423–2434
9 Vardy, E., Arkin, I. T., Gottschalk, K. E., Kaback, H. R. and Schuldiner, S. (2004) Structural conservation in the major facilitator superfamily as revealed by comparative modeling. Protein Sci. **13**, 1832–1840
10 Varoqui, H. and Erickson, J. D. (1996) Active transport of acetylcholine by the human vesicular acetylcholine transporter. J. Biol. Chem. **271**, 27229–27232
11 Hori, T. and Takahashi, T. (2012) Kinetics of synaptic vesicle refilling with neurotransmitter glutamate. Neuron **76**, 511–517
12 Wess, J. (2012) Novel muscarinic receptor mutant mouse models. Handb. Exp. Pharmacol. **208**, 95–117
13 Wess, J., Eglen, R. M. and Gautam, D. (2007) Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development. Nat. Rev. Drug Discovery **6**, 721–733
14 Wess, J. (2004) Muscarinic acetylcholine receptor knockout mice: novel phenotypes and clinical implications. Annu. Rev. Pharmacol. Toxicol. **44**, 423–450
15 Bymaster, F. P., McKinzie, D. L., Felder, C. C. and Wess, J. (2003) Use of M_1–M_5 muscarinic receptor knockout mice as novel tools to delineate the physiological roles of the muscarinic cholinergic system. Neurochem. Res. **28**, 437–442
16 Changeux, J. P. (2010) Nicotine addiction and nicotinic receptors: lessons from genetically modified mice. Nat. Rev. Neurosci. **11**, 389–401
17 Champtiaux, N. and Changeux, J. P. (2004) Knockout and knockin mice to investigate the role of nicotinic receptors in the central nervous system. Prog. Brain Res. **145**, 235–251
18 Cordero-Erausquin, M., Marubio, L. M., Klink, R. and Changeux, J. P. (2000) Nicotinic receptor function: new perspectives from knockout mice. Trends Pharmacol. Sci. **21**, 211–217
19 Khare, P., Ojeda, A. M., Chandrasekaran, A. and Parsons, S. M. (2010) Possible important pair of acidic residues in vesicular acetylcholine transporter. Biochemistry **49**, 3049–3059
20 Nguyen, M. L., Cox, G. D. and Parsons, S. M. (1998) Kinetic parameters for the vesicular acetylcholine transporter: two protons are exchanged for one acetylcholine. Biochemistry **37**, 13400–13410
21 Ojeda, A. M., Kolmakova, N. G. and Parsons, S. M. (2004) Acetylcholine binding site in the vesicular acetylcholine transporter. Biochemistry **43**, 11163–11174
22 Kim, M. H., Lu, M., Lim, E. J., Chai, Y. G. and Hersh, L. B. (1999) Mutational analysis of aspartate residues in the transmembrane regions and cytoplasmic loops of rat vesicular acetylcholine transporter. J. Biol. Chem. **274**, 673–680
23 Bravo, D. T., Kolmakova, N. G. and Parsons, S. M. (2005) Mutational and pH analysis of ionic residues in transmembrane domains of vesicular acetylcholine transporter. Biochemistry **44**, 7955–7966
24 Van der Kloot, W. (2003) Loading and recycling of synaptic vesicles in the Torpedo electric organ and the vertebrate neuromuscular junction. Prog. Neurobiol. **71**, 269–303
25 Song, H., Ming, G., Fon, E., Bellocchio, E., Edwards, R. H. and Poo, M. (1997) Expression of a putative vesicular acetylcholine transporter facilitates quantal transmitter packaging. Neuron **18**, 815–826
26 Prado, V. F., Martins-Silva, C., de Castro, B. M., Lima, R. F., Barros, D. M., Amaral, E., Ramsey, A. J., Sotnikova, T. D., Ramirez, M. R., Kim, H. G. et al. (2006) Mice deficient for the vesicular acetylcholine transporter are myasthenic and have deficits in object and social recognition. Neuron **51**, 601–612
27 Lima, R. F., Prado, V. F., Prado, M. A. and Kushmerick, C. (2010) Quantal release of acetylcholine in mice with reduced levels of the vesicular acetylcholine transporter. J. Neurochem. **113**, 943–951
28 Prior, C., Marshall, I. G. and Parsons, S. M. (1992) The pharmacology of vesamicol: an inhibitor of the vesicular acetylcholine transporter. Gen. Pharmacol. **23**, 1017–1022
29 Tu, Z., Wang, W., Cui, J., Zhang, X., Lu, X., Xu, J. and Parsons, S. M. (2012) Synthesis and evaluation of *in vitro* bioactivity for vesicular acetylcholine transporter inhibitors containing two carbonyl groups. Bioorg. Med. Chem. **20**, 4422–4429
30 Efange, S. M., Khare, A. B., von, H. K., Mach, R. H., Parsons, S. M. and Tu, Z. (2010) Synthesis and *in vitro* biological evaluation of carbonyl group-containing inhibitors of vesicular acetylcholine transporter. J. Med. Chem. **53**, 2825–2835
31 Gras, C., Amilhon, B., Lepicard, E. M., Poirel, O., Vinatier, J., Herbin, M., Dumas, S., Tzavara, E. T., Wade, M. R., Nomikos, G. G. et al. (2008) The vesicular glutamate transporter VGLUT3 synergizes striatal acetylcholine tone. Nat. Neurosci. **11**, 292–300
32 Hnasko, T. S. and Edwards, R. H. (2012) Neurotransmitter corelease: mechanism and physiological role. Annu. Rev. Physiol. **74**, 225–243
33 Trudeau, L. E. and Gutierrez, R. (2007) On cotransmission and neurotransmitter phenotype plasticity. Mol. Interv. **7**, 138–146
34 Mendez, J. A., Bourque, M. J., Dal Bo, G., Bourdeau, M. L., Danik, M., Williams, S., Lacaille, J. C. and Trudeau, L. E. (2008) Developmental and target-dependent regulation of vesicular glutamate transporter expression by dopamine neurons. J. Neurosci. **28**, 6309–6318
35 Tritsch, N. X., Ding, J. B. and Sabatini, B. L. (2012) Dopaminergic neurons inhibit striatal output through non-canonical release of GABA. Nature **490**, 262–266
36 Bravo, D. T., Kolmakova, N. G. and Parsons, S. M. (2004) Choline is transported by vesicular acetylcholine transporter. J. Neurochem. **91**, 766–768

37 Bravo, D. T., Kolmakova, N. G. and Parsons, S. M. (2005) New transport assay demonstrates vesicular acetylcholine transporter has many alternative substrates. Neurochem. Int. **47**, 243–247

38 Khare, P., Mulakaluri, A. and Parsons, S. M. (2010) Search for the acetylcholine and vesamicol binding sites in vesicular acetylcholine transporter: the region around the lumenal end of the transport channel. J. Neurochem. **115**, 984–993

39 Barbosa, Jr, J., Clarizia, A. D., Gomez, M. V., RomanoSilva, M. A., Prado, V. F. and Prado, M. A. M. (1997) Effect of protein kinase C activation on the release of [^{3}H]Acetylcholine in the presence of vesamicol. J. Neurochem. **69**, 2608–2611

40 Clarizia, A. D., Gomez, M. V., Romano-Silva, M. A., Parsons, S. M., Prado, V. F. and Prado, M. A. (1999) Control of the binding of a vesamicol analog to the vesicular acetylcholine transporter. NeuroReport **10**, 2783–2787

41 Clarizia, A. D., Romano-Silva, M. A., Prado, V. F., Gomez, M. V. and Prado, M. A. M. (1998) Role of protein kinase C in the release of [^{3}H]acetylcholine from myenteric plexus treated with vesamicol. Neurosci. Lett. **244**, 115–117

42 Efange, S. M., von Hohenberg, K., Khare, A. B., Tu, Z., Mach, R. H. and Parsons, S. M. (2000) Synthesis and biological characterization of stable and radioiodinated (±)-*trans*-2-hydroxy-3-{4-(3-iodophenyl)piperidyl}-1,2,3,4-tetrahydronaphthalene (3′-IBVM). Nucl. Med. Biol. **27**, 749–755

43 Tu, Z., Efange, S. M., Xu, J., Li, S., Jones, L. A., Parsons, S. M. and Mach, R. H. (2009) Synthesis and *in vitro* and *in vivo* evaluation of 18F-labeled positron emission tomography (PET) ligands for imaging the vesicular acetylcholine transporter. J. Med. Chem. **52**, 1358–1369

44 Kozaka, T., Uno, I., Kitamura, Y., Miwa, D., Ogawa, K. and Shiba, K. (2012) Syntheses and *in vitro* evaluation of decalinvesamicol analogues as potential imaging probes for vesicular acetylcholine transporter (VAChT). Bioorg. Med. Chem. **20**, 4936–4941

45 Grygoruk, A., Fei, H., Daniels, R. W., Miller, B. R., Chen, A., DiAntonio, A. and Krantz, D. E. (2010) Vesicular neurotransmitter transporter trafficking *in vivo*: moving from cells to flies. Fly **4**, 302–305

46 Prado, V. F. and Prado, M. A. (2002) Signals involved in targeting membrane proteins to synaptic vesicles. Cell. Mol. Neurobiol. **22**, 565–577

47 Gilmor, M. L., Nash, N. R., Roghani, A., Edwards, R. H., Yi, H., Hersch, S. M. and Levey, A. I. (1996) Expression of the putative vesicular acetylcholine transporter in rat brain and localization in cholinergic synaptic vesicles. J. Neurosci. **16**, 2179–2190

48 Liu, Y. and Edwards, R. H. (1997) The role of vesicular transport proteins in synaptic transmission and neural degeneration. Annu. Rev. Neurosci. **20**, 125–156

49 Krantz, D. E., Waites, C., Oorschot, V., Liu, Y., Wilson, R. I., Tan, P. K., Klumperman, J. and Edwards, R. H. (2000) A phosphorylation site regulates sorting of the vesicular acetylcholine transporter to dense core vesicles. J. Cell Biol. **149**, 379–396

50 Varoqui, H. and Erickson, J. D. (1998) The cytoplasmic tail of the vesicular acetylcholine transporter contains a synaptic vesicle targeting signal. J. Biol. Chem. **273**, 9094–9098

51 Tan, P. K., Waites, C., Liu, Y., Krantz, D. E. and Edwards, R. H. (1998) A leucine-based motif mediates the endocytosis of vesicular monoamine and acetylcholine transporters. J. Biol. Chem. **273**, 17351–17360

52 Barbosa, Jr, J., Ferreira, L. T., Martins-Silva, C., Santos, M. S., Torres, G. E., Caron, M. G., Gomez, M. V., Ferguson, S. S., Prado, M. A. and Prado, V. F. (2002) Trafficking of the vesicular acetylcholine transporter in SN56 cells: a dynamin-sensitive step and interaction with the AP-2 adaptor complex. J. Neurochem. **82**, 1221–1228

53 Ferreira, L. T., Santos, M. S., Kolmakova, N. G., Koenen, J., Barbosa, Jr, J., Gomez, M. V., Guatimosim, C., Zhang, X., Parsons, S. M., Prado, V. F. and Prado, M. A. (2005) Structural requirements for steady-state localization of the vesicular acetylcholine transporter. J. Neurochem. **94**, 957–969

54 Santos, M. S., Barbosa, J., Kushmerick, C., Gomez, M. V., Ferguson, S. S. G., Prado, V. F. and Prado, M. A. M. (2001) Visualization and trafficking of the vesicular acetylcholine transporter in living cholinergic cells. J. Neurochem. **78**, 37–37

55 Colgan, L., Liu, H., Huang, S. Y. and Liu, Y. J. (2007) Dileucine motif is sufficient for internalization and synaptic vesicle targeting of vesicular acetylcholine transporter. Traffic **8**, 512–522

56 Ribeiro, F. M., Alves-Silva, J., Volknandt, W., Martins-Silva, C., Mahmud, H., Wilhelm, A., Gomez, M. V., Rylett, R. J., Ferguson, S. S., Prado, V. F. and Prado, M. A. (2003) The hemicholinium-3 sensitive high affinity choline transporter is internalized by clathrin-mediated endocytosis and is present in endosomes and synaptic vesicles. J. Neurochem. **87**, 136–146

57 Ribeiro, F. M., Black, S. A., Cregan, S. P., Prado, V. F., Prado, M. A., Rylett, R. J. and Ferguson, S. S. (2005) Constitutive high-affinity choline transporter endocytosis is determined by a carboxyl-terminal tail dileucine motif. J. Neurochem. **94**, 86–96

58 Ribeiro, F. M., Pinthong, M., Black, S. A., Gordon, A. C., Prado, V. F., Prado, M. A., Rylett, R. J. and Ferguson, S. S. (2007) Regulated recycling and plasma membrane recruitment of the high-affinity choline transporter. Eur. J. Neurosci. **26**, 3437–3448

59 Sandoval, G. M., Duerr, J. S., Hodgkin, J., Rand, J. B. and Ruvkun, G. (2006) A genetic interaction between the vesicular acetylcholine transporter VAChT/UNC-17 and synaptobrevin/SNB-1 in *C. elegans*. Nat. Neurosci. **9**, 599–601

60 Ribeiro, F. M., Ferreira, L. T., Marion, S., Fontes, S., Gomez, M., Ferguson, S. S., Prado, M. A. and Prado, V. F. (2007) SEC14-like protein 1 interacts with cholinergic transporters. Neurochem. Int. **50**, 356–364

61 Terry, Jr, A., Gearhart, D. A., Warner, S. E., Zhang, G., Bartlett, M. G., Middlemore, M. L., Beck, Jr, W. D., Mahadik, S. P. and Waller, J. L. (2007) Oral haloperidol or risperidone treatment in rats: temporal effects on nerve growth factor receptors, cholinergic neurons, and memory performance. Neuroscience **146**, 1316–1332

62 Chen, K. H., Reese, E. A., Kim, H. W., Rapoport, S. I. and Rao, J. S. (2011) Disturbed neurotransmitter transporter expression in Alzheimer's disease brain. J. Alzheimers Dis. **26**, 755–766

63 Efange, S. M., Garland, E. M., Staley, J. K., Khare, A. B. and Mash, D. C. (1997) Vesicular acetylcholine transporter density and Alzheimer's disease. Neurobiol. Aging **18**, 407–413

64 Semmler, A., Frisch, C., Debeir, T., Ramanathan, M., Okulla, T., Klockgether, T. and Heneka, M. T. (2007) Long-term cognitive impairment, neuronal loss and reduced cortical cholinergic innervation after recovery from sepsis in a rodent model. Exp. Neurol. **204**, 733–740

65 Li, D. J., Evans, R. G., Yang, Z. W., Song, S. W., Wang, P., Ma, X. J., Liu, C., Xi, T., Su, D. F. and Shen, F. M. (2011) Dysfunction of the cholinergic anti-inflammatory pathway mediates organ damage in hypertension. Hypertension **57**, 298–307

66 Smith, R., Chung, H., Rundquist, S., Maat-Schieman, M. L., Colgan, L., Englund, E., Liu, Y. J., Roos, R. A., Faull, R. L., Brundin, P. and Li, J. Y. (2006) Cholinergic neuronal defect without cell loss in Huntington's disease. Hum. Mol. Genet. **15**, 3119–3131

67 Edwards, R. H. (2007) The neurotransmitter cycle and quantal size. Neuron **55**, 835–858

68 Contant, C., Umbriaco, D., Garcia, S., Watkins, K. C. and Descarries, L. (1996) Ultrastructural characterization of the acetylcholine innervation in adult rat neostriatum. Neuroscience **71**, 937–947

69 Duffy, A. M., Zhou, P., Milner, T. A. and Pickel, V. M. (2009) Spatial and intracellular relationships between the alpha7 nicotinic acetylcholine receptor and the vesicular acetylcholine transporter in the prefrontal cortex of rat and mouse. Neuroscience **161**, 1091–1103

70 Nagy, P. M. and Aubert, I. (2012) Overexpression of the vesicular acetylcholine transporter increased acetylcholine release in the hippocampus. Neuroscience **218**, 1–11

71 de Castro, B. M., De Jaeger, X., Martins-Silva, C., Lima, R. F., Amaral, E., Menezes, C., Lima, P., Neves, C. M. L., Pires, R. G., Gould, T. W. et al. (2009) The vesicular acetylcholine transporter is required for neuromuscular development and function. Mol. Cell. Biol. **29**, 5238–5250

72 de Castro, B. M., Pereira, G. S., Magalhaes, V., Rossato, J. I., De Jaeger, X., Martins-Silva, C., Leles, B., Lima, P., Gomez, M. V., Gainetdinov, R. R. et al. (2009) Reduced expression of the vesicular acetylcholine transporter causes learning deficits in mice. Genes Brain Behav. **8**, 23–35

73 Lara, A., Damasceno, D. D., Pires, R., Gros, R., Gomes, E. R., Gavioli, M., Lima, R. F., Guimaraes, D., Lima, P., Bueno, Jr, C. R. et al. (2010) Dysautonomia due to reduced cholinergic neurotransmission causes cardiac remodeling and heart failure. Mol. Cell. Biol. **30**, 1746–1756

74 Martins-Silva, C., De Jaeger, X., Guzman, M. S., Lima, R. D., Santos, M. S., Kushmerick, C., Gomez, M. V., Caron, M. G., Prado, M. A. and Prado, V. F. (2011) Novel strains of mice deficient for the vesicular acetylcholine transporter: insights on transcriptional regulation and control of locomotor behavior. PLoS ONE **6**, e17611

75 Martyn, A. C., De Jaeger, X., Magalhaes, A. C., Kesarwani, R., Goncalves, D. F., Raulic, S., Guzman, M. S., Jackson, M. F., Izquierdo, I., Macdonald, J. F. et al. (2012) Elimination of the vesicular acetylcholine transporter in the forebrain causes hyperactivity and deficits in spatial memory and long-term potentiation. Proc. Natl. Acad. Sci. U.S.A. **109**, 17651–17656

76 Guzman, M. S., De Jaeger, X., Raulic, S., Souza, I. A., Li, A. X., Schmid, S., Menon, R. S., Gainetdinov, R. R., Caron, M. G., Bartha, R. et al. (2011) Elimination of the vesicular acetylcholine transporter in the striatum reveals regulation of behaviour by cholinergic-glutamatergic co-transmission. PLoS Biol. **9**, e1001194

77 Liu, Y., Peter, D., Roghani, A., Schuldiner, S., Prive, G. G., Eisenberg, D., Brecha, N. and Edwards, R. H. (1992) A cDNA that suppresses MPP^+ toxicity encodes a vesicular amine transporter. Cell **70**, 539–551

78 Misgeld, T., Burgess, R. W., Lewis, R. M., Cunningham, J. M., Lichtman, J. W. and Sanes, J. R. (2002) Roles of neurotransmitter in synapse formation: development of neuromuscular junctions lacking choline acetyltransferase. Neuron **36**, 635–648

79 Brandon, E. P., Lin, W., D'Amour, K. A., Pizzo, D. P., Dominguez, B., Sugiura, Y., Thode, S., Ko, C. P., Thal, L. J., Gage, F. H. and Lee, K. F. (2003) Aberrant patterning of neuromuscular synapses in choline acetyltransferase-deficient mice. J. Neurosci. **23**, 539–549

80 De Jaeger, X., Cammarota, M., Prado, M. A., Izquierdo, I., Prado, V. F. and Pereira, G. S. (2012) Decreased acetylcholine release delays the consolidation of object recognition memory. Behav. Brain Res. **238C**, 62–68

81 Takamori, S., Holt, M., Stenius, K., Lemke, E. A., Gronborg, M., Riedel, D., Urlaub, H., Schenck, S., Brugger, B., Ringler, P. et al. (2006) Molecular anatomy of a trafficking organelle. Cell **127**, 831–846

82 Hasselmo, M. E. and Sarter, M. (2011) Modes and models of forebrain cholinergic neuromodulation of cognition. Neuropsychopharmacology **36**, 52–73

83 Sarter, M. and Paolone, G. (2011) Deficits in attentional control: cholinergic mechanisms and circuitry-based treatment approaches. Behav. Neurosci. **125**, 825–835

84 Howe, W. M., Ji, J., Parikh, V., Williams, S., Mocaer, E., Trocme-Thibierge, C. and Sarter, M. (2010) Enhancement of attentional performance by selective stimulation of alpha4beta2(*) nAChRs: underlying cholinergic mechanisms. Neuropsychopharmacology **35**, 1391–1401

85 Parikh, V. and Sarter, M. (2008) Cholinergic mediation of attention: contributions of phasic and tonic increases in prefrontal cholinergic activity. Ann. N.Y. Acad. Sci. **1129**, 225–235

86 Hasselmo, M. E. and Giocomo, L. M. (2006) Cholinergic modulation of cortical function. J. Mol. Neurosci. **30**, 133–135

87 Hasselmo, M. E. and Stern, C. E. (2006) Mechanisms underlying working memory for novel information. Trends Cogn. Sci. **10**, 487–493

88 Hasselmo, M. E. (2006) The role of acetylcholine in learning and memory. Curr. Opin. Neurobiol. **16**, 710–715

89 Ren, J., Qin, C., Hu, F., Tan, J., Qiu, L., Zhao, S., Feng, G. and Luo, M. (2011) Habenula 'cholinergic' neurons corelease glutamate and acetylcholine and activate postsynaptic neurons via distinct transmission modes. Neuron **69**, 445–452

90 Nickerson Poulin, A., Guerci, A., El Mestikawy, S. and Semba, K. (2006) Vesicular glutamate transporter 3 immunoreactivity is present in cholinergic basal forebrain neurons projecting to the basolateral amygdala in rat. J. Comp. Neurol. **498**, 690–711

91 Schafer, M. K., Varoqui, H., Defamie, N., Weihe, E. and Erickson, J. D. (2002) Molecular cloning and functional identification of mouse vesicular glutamate transporter 3 and its expression in subsets of novel excitatory neurons. J. Biol. Chem. **277**, 50734–50748

92 Lee, S., Kim, K. and Zhou, Z. J. (2010) Role of ACh-GABA cotransmission in detecting image motion and motion direction. Neuron **68**, 1159–1172

93 Higley, M. J., Gittis, A. H., Oldenburg, I. A., Balthasar, N., Seal, R. P., Edwards, R. H., Lowell, B. B., Kreitzer, A. C. and Sabatini, B. L. (2011) Cholinergic interneurons mediate fast VGluT3-dependent glutamatergic transmission in the striatum. PLoS ONE **6**, e19155

94 Hikida, T., Kaneko, S., Isobe, T., Kitabatake, Y., Watanabe, D., Pastan, I. and Nakanishi, S. (2001) Increased sensitivity to cocaine by cholinergic cell ablation in nucleus accumbens. Proc. Natl. Acad. Sci. U.S.A. **98**, 13351–13354

95 Kitabatake, Y., Hikida, T., Watanabe, D., Pastan, I. and Nakanishi, S. (2003) Impairment of reward-related learning by cholinergic cell ablation in the striatum. Proc. Natl. Acad. Sci. U.S.A. **100**, 7965–7970

96 Threlfell, S., Lalic, T., Platt, N. J., Jennings, K. A., Deisseroth, K. and Cragg, S. J. (2012) Striatal dopamine release is triggered by synchronized activity in cholinergic interneurons. Neuron **75**, 58–64

97 Cachope, R., Mateo, Y., Mathur, B. N., Irving, J., Wang, H. L., Morales, M., Lovinger, D. M. and Cheer, J. F. (2012) Selective activation of cholinergic interneurons enhances accumbal phasic dopamine release: setting the tone for reward processing. Cell Rep. **2**, 33–41

98 English, D. F., Ibanez-Sandoval, O., Stark, E., Tecuapetla, F., Buzsaki, G., Deisseroth, K., Tepper, J. M. and Koos, T. (2012) GABAergic circuits mediate the reinforcement-related signals of striatal cholinergic interneurons. Nat. Neurosci. **15**, 123–130

99 Gallagher, M. and Colombo, P. J. (1995) Ageing: the cholinergic hypothesis of cognitive decline. Curr. Opin. Neurobiol. **5**, 161–168

100 Mesulam, M. (2004) The cholinergic lesion of Alzheimer's disease: pivotal factor or side show? Learn. Mem. **11**, 43–49

101 Mesulam, M., Shaw, P., Mash, D. and Weintraub, S. (2004) Cholinergic nucleus basalis tauopathy emerges early in the aging-MCI-AD continuum. Ann. Neurol. **55**, 815–828

102 Schliebs, R. and Arendt, T. (2006) The significance of the cholinergic system in the brain during aging and in Alzheimer's disease. J. Neural Transm. **113**, 1625–1644

103 Schliebs, R., Rossner, S. and Bigl, V. (1996) Immunolesion by 192IgG-saporin of rat basal forebrain cholinergic system: a useful tool to produce cortical cholinergic dysfunction. Prog. Brain Res. **109**, 253–264

104 Berson, A., Barbash, S., Shaltiel, G., Goll, Y., Hanin, G., Greenberg, D. S., Ketzef, M., Becker, A. J., Friedman, A. and Soreq, H. (2012) Cholinergic-associated loss of hnRNP-A/B in Alzheimer's disease impairs cortical splicing and cognitive function in mice. EMBO Mol. Med. **4**, 730–742

105 Arvidsson, U., Riedl, M., Elde, R. and Meister, B. (1997) Vesicular acetylcholine transporter (VAChT) protein: a novel and unique marker for cholinergic neurons in the central and peripheral nervous systems. J. Comp. Neurol. **378**, 454–467

106 Olshansky, B., Sabbah, H. N., Hauptman, P. J. and Colucci, W. S. (2008) Parasympathetic nervous system and heart failure: pathophysiology and potential implications for therapy. Circulation **118**, 863–871

107 Hasking, G. J., Esler, M. D., Jennings, G. L., Burton, D., Johns, J. A. and Korner, P. I. (1986) Norepinephrine spillover to plasma in patients with congestive heart failure: evidence of increased overall and cardiorenal sympathetic nervous activity. Circulation **73**, 615–621

108 Nolan, J., Flapan, A. D., Capewell, S., MacDonald, T. M., Neilson, J. M. and Ewing, D. J. (1992) Decreased cardiac parasympathetic activity in chronic heart failure and its relation to left ventricular function. Br. Heart J. **67**, 482–485

109 Floras, J. S. (1993) Clinical aspects of sympathetic activation and parasympathetic withdrawal in heart failure. J. Am. Coll. Cardiol. **22**, 72A–84A

110 Borovikova, L. V., Ivanova, S., Zhang, M., Yang, H., Botchkina, G. I., Watkins, L. R., Wang, H., Abumrad, N., Eaton, J. W. and Tracey, K. J. (2000) Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature **405**, 458–462

111 Kawada, T., Yamazaki, T., Akiyama, T., Li, M., Ariumi, H., Mori, H., Sunagawa, K. and Sugimachi, M. (2006) Vagal stimulation suppresses ischemia-induced myocardial interstitial norepinephrine release. Life Sci. **78**, 882–887

112 Kawada, T., Yamazaki, T., Akiyama, T., Shishido, T., Inagaki, M., Uemura, K., Miyamoto, T., Sugimachi, M., Takaki, H. and Sunagawa, K. (2001) *In vivo* assessment of acetylcholine-releasing function at cardiac vagal nerve terminals. Am. J. Physiol. Heart Circ. Physiol. **281**, H139–H145

113 Kessler, W., Traeger, T., Westerholt, A., Neher, F., Mikulcak, M., Muller, A., Maier, S. and Heidecke, C. D. (2006) The vagal nerve as a link between the nervous and immune system in the instance of polymicrobial sepsis. Langenbecks Arch. Surg. **391**, 83–87

114 Li, M., Zheng, C., Sato, T., Kawada, T., Sugimachi, M. and Sunagawa, K. (2004) Vagal nerve stimulation markedly improves long-term survival after chronic heart failure in rats. Circulation **109**, 120–124

115 Lopez, N. E., Krzyzaniak, M., Costantini, T. W., De, M. A., Baird, A., Eliceiri, B. P. and Coimbra, R. (2012) Vagal nerve stimulation blocks peritoneal macrophage inflammatory responsiveness after severe burn injury. Shock **38**, 294–300

116 Tsutsumi, T., Ide, T., Yamato, M., Kudou, W., Andou, M., Hirooka, Y., Utsumi, H., Tsutsui, H. and Sunagawa, K. (2008) Modulation of the myocardial redox state by vagal nerve stimulation after experimental myocardial infarction. Cardiovasc. Res. **77**, 713–721

117 Uemura, K., Li, M., Tsutsumi, T., Yamazaki, T., Kawada, T., Kamiya, A., Inagaki, M., Sunagawa, K. and Sugimachi, M. (2007) Efferent vagal nerve stimulation induces tissue inhibitor of metalloproteinase-1 in myocardial ischemia-reperfusion injury in rabbit. Am. J. Physiol. Heart Circ. Physiol. **293**, H2254–H2261

118 Zheng, C., Li, M., Inagaki, M., Kawada, T., Sunagawa, K. and Sugimachi, M. (2005) Vagal stimulation markedly suppresses arrhythmias in conscious rats with chronic heart failure after myocardial infarction. Conf. Proc. IEEE Eng. Med. Biol. Soc. **7**, 7072–7075

119 Roy, A., Lara, A., Guimaraes, D., Pires, R., Gomes, E. R., Carter, D. E., Gomez, M. V., Guatimosim, S., Prado, V. F., Prado, M. A. and Gros, R. (2012) An analysis of the myocardial transcriptome in a mouse model of cardiac dysfunction with decreased cholinergic neurotransmission. PLoS ONE **7**, e39997

120 LaCroix, C., Freeling, J., Giles, A., Wess, J. and Li, Y. F. (2008) Deficiency of M_2 muscarinic acetylcholine receptors increases susceptibility of ventricular function to chronic adrenergic stress. Am. J. Physiol. Heart Circ. Physiol. **294**, H810–H820

121 English, B. A., Appalsamy, M., Diedrich, A., Ruggiero, A. M., Lund, D., Wright, J., Keller, N. R., Louderback, K. M., Robertson, D. and Blakely, R. D. (2010) Tachycardia, reduced vagal capacity, and age-dependent ventricular dysfunction arising from diminished expression of the presynaptic choline transporter. Am. J. Physiol. Heart Circ. Physiol. **299**, H799–H810

122 Tracey, K. J. (2002) The inflammatory reflex. Nature **420**, 853–859

123 Wang, H., Yu, M., Ochani, M., Amella, C. A., Tanovic, M., Susarla, S., Li, J. H., Wang, H., Yang, H., Ulloa, L. et al. (2003) Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. Nature **421**, 384–388

124 Mann, D. L. (2002) Inflammatory mediators and the failing heart: past, present, and the foreseeable future. Circ. Res. **91**, 988–998

125 Wang, H., Liao, H., Ochani, M., Justiniani, M., Lin, X., Yang, L., Al Abed, Y., Wang, H., Metz, C., Miller, E. J. et al. (2004) Cholinergic agonists inhibit HMGB1 release and improve survival in experimental sepsis. Nat. Med. **10**, 1216–1221

126 Pavlov, V. A., Ochani, M., Yang, L. H., Gallowitsch-Puerta, M., Ochani, K., Lin, X., Levi, J., Parrish, W. R., Rosas-Ballina, M., Czura, C. J. et al. (2007) Selective alpha7-nicotinic acetylcholine receptor agonist GTS-21 improves survival in murine endotoxemia and severe sepsis. Crit. Care Med. **35**, 1139–1144

127 van Westerloo, D. J., Giebelen, I. A., Florquin, S., Daalhuisen, J., Bruno, M. J., de Vos, A. F., Tracey, K. J. and van der Poll, T. (2005) The cholinergic anti-inflammatory pathway regulates the host response during septic peritonitis. J. Infect. Dis. **191**, 2138–2148

128 Ribeiro Machado, M. P., Rocha, A. M., Oliveira, L. F., Cuba, M. B., Loss, I. O., Castellano, L. C., Silva, M. V., Machado, J. R., Nascentes, G. A., Paiva, L. H. et al. (2012) Autonomic nervous system modulation affects the inflammatory immune response in mice with acute Chagas disease. Exp. Physiol. **97**, 1186–1202

129 Masland, R. H., Mills, J. W. and Cassidy, C. (1984) The functions of acetylcholine in the rabbit retina. Proc. R. Soc. London Ser. B **223**, 121–139
130 Furness, J. B. (2000) Types of neurons in the enteric nervous system. J. Auton. Nerv. Syst. **81**, 87–96
131 Bornstein, J. C., Costa, M. and Grider, J. R. (2004) Enteric motor and interneuronal circuits controlling motility. Neurogastroenterol. Motil. **16**, 34–38
132 Barnes, P. J. (2004) Distribution of receptor targets in the lung. Proc. Am. Thorac. Soc. **1**, 345–351
133 Wessler, I., Kirkpatrick, C. J. and Racke, K. (1998) Non-neuronal acetylcholine, a locally acting molecule, widely distributed in biological systems: expression and function in humans. Pharmacol. Ther. **77**, 59–79
134 Kawashima, K. and Fujii, T. (2000) Extraneuronal cholinergic system in lymphocytes. Pharmacol. Ther. **86**, 29–48
135 Fujii, T. and Kawashima, K. (2001) An independent non-neuronal cholinergic system in lymphocytes. Jpn. J. Pharmacol. **85**, 11–15
136 Reinheimer, T., Bernedo, P., Klapproth, H., Oelert, H., Zeiske, B., Racke, K. and Wessler, I. (1996) Acetylcholine in isolated airways of rat, guinea pig, and human: species differences in role of airway mucosa. Am. J. Physiol. **270**, L722–L728
137 Proskocil, B. J., Sekhon, H. S., Jia, Y., Savchenko, V., Blakely, R. D., Lindstrom, J. and Spindel, E. R. (2004) Acetylcholine is an autocrine or paracrine hormone synthesized and secreted by airway bronchial epithelial cells. Endocrinology **145**, 2498–2506
138 Klapproth, H., Reinheimer, T., Metzen, J., Munch, M., Bittinger, F., Kirkpatrick, C. J., Hohle, K. D., Schemann, M., Racke, K. and Wessler, I. (1997) Non-neuronal acetylcholine, a signalling molecule synthezised by surface cells of rat and man. Naunyn Schmiedebergs Arch. Pharmacol. **355**, 515–523
139 Kawashima, K., Watanabe, N., Oohata, H., Fujimoto, K., Suzuki, T., Ishizaki, Y., Morita, I. and Murota, S. (1990) Synthesis and release of acetylcholine by cultured bovine arterial endothelial cells. Neurosci. Lett. **119**, 156–158
140 Kakinuma, Y., Akiyama, T. and Sato, T. (2009) Cholinoceptive and cholinergic properties of cardiomyocytes involving an amplification mechanism for vagal efferent effects in sparsely innervated ventricular myocardium. FEBS J. **276**, 5111–5125
141 Rana, O. R., Schauerte, P., Kluttig, R., Schroder, J. W., Koenen, R. R., Weber, C., Nolte, K. W., Weis, J., Hoffmann, R., Marx, N. and Saygili, E. (2010) Acetylcholine as an age-dependent non-neuronal source in the heart. Auton. Neurosci. **156**, 82–89
142 Kawashima, K. and Fujii, T. (2003) The lymphocytic cholinergic system and its contribution to the regulation of immune activity. Life Sci. **74**, 675–696
143 Kawashima, K. and Fujii, T. (2004) Expression of non-neuronal acetylcholine in lymphocytes and its contribution to the regulation of immune function. Front. Biosci. **9**, 2063–2085
144 Rosas-Ballina, M., Olofsson, P. S., Ochani, M., Valdes-Ferrer, S. I., Levine, Y. A., Reardon, C., Tusche, M. W., Pavlov, V. A., Andersson, U., Chavan, S. et al. (2011) Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. Science **334**, 98–101
145 Kawashima, K., Yoshikawa, K., Fujii, Y. X., Moriwaki, Y. and Misawa, H. (2007) Expression and function of genes encoding cholinergic components in murine immune cells. Life Sci. **80**, 2314–2319
146 Lips, K. S., Volk, C., Schmitt, B. M., Pfeil, U., Arndt, P., Miska, D., Ermert, L., Kummer, W. and Koepsell, H. (2005) Polyspecific cation transporters mediate luminal release of acetylcholine from bronchial epithelium. Am. J. Respir. Cell Mol. Biol. **33**, 79–88
147 Kummer, W., Wiegand, S., Akinci, S., Wessler, I., Schinkel, A. H., Wess, J., Koepsell, H., Haberberger, R. V. and Lips, K. S. (2006) Role of acetylcholine and polyspecific cation transporters in serotonin-induced bronchoconstriction in the mouse. Respir. Res. **7**, 65
148 Metzen, J., Bittinger, F., Kirkpatrick, C. J., Kilbinger, H. and Wessler, I. (2003) Proliferative effect of acetylcholine on rat trachea epithelial cells is mediated by nicotinic receptors and muscarinic receptors of the M_1-subtype. Life Sci. **72**, 2075–2080
149 Improgo, M. R., Tapper, A. R. and Gardner, P. D. (2011) Nicotinic acetylcholine receptor-mediated mechanisms in lung cancer. Biochem. Pharmacol. **82**, 1015–1021
150 Pettersson, A., Nilsson, L., Nylund, G., Khorram-Manesh, A., Nordgren, S. and Delbro, D. S. (2009) Is acetylcholine an autocrine/paracrine growth factor via the nicotinic alpha7-receptor subtype in the human colon cancer cell line HT-29? Eur. J. Pharmacol. **609**, 27–33
151 Cheng, K., Samimi, R., Xie, G., Shant, J., Drachenberg, C., Wade, M., Davis, R. J., Nomikos, G. and Raufman, J. P. (2008) Acetylcholine release by human colon cancer cells mediates autocrine stimulation of cell proliferation. Am. J. Physiol. Gastrointest. Liver Physiol. **295**, G591–G597
152 Gilon, P. and Henquin, J. C. (2001) Mechanisms and physiological significance of the cholinergic control of pancreatic beta-cell function. Endocr. Rev. **22**, 565–604
153 Gautam, D., Gavrilova, O., Jeon, J., Pack, S., Jou, W., Cui, Y., Li, J. H. and Wess, J. (2006) Beneficial metabolic effects of M_3 muscarinic acetylcholine receptor deficiency. Cell Metab. **4**, 363–375
154 Furchgott, R. F. and Zawadzki, J. V. (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature **288**, 373–376
155 Ikeda, C., Morita, I., Mori, A., Fujimoto, K., Suzuki, T., Kawashima, K. and Murota, S. (1994) Phorbol ester stimulates acetylcholine synthesis in cultured endothelial cells isolated from porcine cerebral microvessels. Brain Res. **655**, 147–152
156 Kirkpatrick, C. J., Bittinger, F., Unger, R. E., Kriegsmann, J., Kilbinger, H. and Wessler, I. (2001) The non-neuronal cholinergic system in the endothelium: evidence and possible pathobiological significance. Jpn. J. Pharmacol. **85**, 24–28
157 Haberberger, R. V., Bodenbenner, M. and Kummer, W. (2000) Expression of the cholinergic gene locus in pulmonary arterial endothelial cells. Histochem. Cell Biol. **113**, 379–387
158 Hoover, D. B., Ganote, C. E., Ferguson, S. M., Blakely, R. D. and Parsons, R. L. (2004) Localization of cholinergic innervation in guinea pig heart by immunohistochemistry for high-affinity choline transporters. Cardiovasc. Res. **62**, 112–121
159 Crick, S. J., Wharton, J., Sheppard, M. N., Royston, D., Yacoub, M. H., Anderson, R. H. and Polak, J. M. (1994) Innervation of the human cardiac conduction system. A quantitative immunohistochemical and histochemical study. Circulation **89**, 1697–1708
160 Okuda, T., Osawa, C., Yamada, H., Hayashi, K., Nishikawa, S., Ushio, T., Kubo, Y., Satou, M., Ogawa, H. and Haga, T. (2012) Transmembrane topology and oligomeric structure of the high-affinity choline transporter. J. Biol. Chem., doi:10.1074/jbc.M112.405027
161 Loewi, O. (1921) Über humorale Übertragbarkeit der Herznervenwirkung. I. Pflügers Archiv. **189**, 239–242

Received 1 November 2012/15 November 2012; accepted 22 November 2012
Published on the Internet 15 February 2013, doi:10.1042/BJ20121662

Appendix B

Hyperactivity and attention deficits in mice with decreased levels of stress inducible phosphoprotein 1 (STIP1)

This is a copyedited, author-produced PDF of an article accepted for publication in *Disease Model Mechanisms* following peer review.

Beraldo FH, Thomas A, Kolisnyk B, Hirata PH, De Jaeger X, Martyn AC, Fan J, Goncalves DF, Cowan MF, Masood T, Martins VR, Gros R, Prado VF, Prado MA. Hyperactivity and attention deficits in mice with decreased levels of stress inducible phosphoprotein 1 (STIP1). Dis Model Mech. 2015 Sep 17

Contributions to publication: BK performed self-grooming behaviour analyses.

© 2015. Published by The Company of Biologists Ltd | Disease Models & Mechanisms (2015) 8, 1457-1466 doi:10.1242/dmm.022525

RESEARCH ARTICLE

Hyperactivity and attention deficits in mice with decreased levels of stress-inducible phosphoprotein 1 (STIP1)

Flavio H. Beraldo[1,*], Anu Thomas[1], Benjamin Kolisnyk[1,2], Pedro H. Hirata[1], Xavier De Jaeger[1], Amanda C. Martyn[1], Jue Fan[1], Daniela F. Goncalves[1], Matthew F. Cowan[1], Talal Masood[1,2], Vilma R. Martins[3], Robert Gros[1,4], Vania F. Prado[1,2,4,5,*] and Marco A. M. Prado[1,2,4,5,*]

ABSTRACT

Stress-inducible phosphoprotein I (STIP1, STI1 or HOP) is a co-chaperone intermediating Hsp70/Hsp90 exchange of client proteins, but it can also be secreted to trigger prion protein-mediated neuronal signaling. Some mothers of children with autism spectrum disorders (ASD) present antibodies against certain brain proteins, including antibodies against STIP1. Maternal antibodies can cross the fetus blood-brain barrier during pregnancy, suggesting the possibility that they can interfere with STIP1 levels and, presumably, functions. However, it is currently unknown whether abnormal levels of STIP1 have any impact in ASD-related behavior. Here, we used mice with reduced (50%) or increased STIP1 levels (fivefold) to test for potential ASD-like phenotypes. We found that increased STIP1 regulates the abundance of Hsp70 and Hsp90, whereas reduced STIP1 does not affect Hsp70, Hsp90 or the prion protein. Interestingly, BAC transgenic mice presenting fivefold more STIP1 show no major phenotype when examined in a series of behavioral tasks, including locomotor activity, elevated plus maze, Morris water maze and five-choice serial reaction time task (5-CSRTT). In contrast, mice with reduced STIP1 levels are hyperactive and have attentional deficits on the 5-CSRTT, but exhibit normal performance for the other tasks. We conclude that reduced STIP1 levels can contribute to phenotypes related to ASD. However, future experiments are needed to define whether it is decreased chaperone capacity or impaired prion protein signaling that contributes to these phenotypes.

KEY WORDS: Touchscreen, Autism, ASD, Stress-inducible phosphoprotein 1, Attention deficits, Mouse model, BAC

INTRODUCTION

In autism spectrum disorders (ASD), alterations in genetic variance and neurodevelopmental are both thought to contribute to phenotype heterogeneity. Womb environment and autoimmune responses have been proposed to contribute to the complex behavioral alterations observed in ASD, which include, but are not limited to, abnormal socialization and communication and stereotyped behavior (Brimberg et al., 2013; Goldani et al., 2014). Several distinct groups have investigated the existence of antibodies against fetal brain tissue in mothers of ASD children (Bauman et al., 2013; Braunschweig et al., 2012b, 2013; Dalton et al., 2003; Nordahl et al., 2013). Passive transfer of maternal anti-brain antibodies to pregnant experimental animal models (including mice, rats and non-human primates) has shown that their offspring develop a number of endophenotypes that resemble phenotypes in ASD (Bauman et al., 2013; Braunschweig et al., 2012b; Dalton et al., 2003). Indeed, a recent study indicated that the prevalence of antibodies against fetal brain proteins is increased fourfold in mothers of an ASD child compared with control groups (Brimberg et al., 2013). Proteomics analysis has identified six brain proteins as targets for ASD antibodies, including lactate dehydrogenase A and B (LDH), cypin, stress-inducible phosphoprotein protein1 (STIP1), collapsine response mediator proteins 1 and 2 (CRMP1, CRMP2) and Y-box-binding protein (YBX1) (Braunschweig et al., 2013). Interestingly, injection of maternal antibodies that recognize LDH, STIP1 and CRMP1 in developing mouse embryos causes an increase in cortical neural precursor proliferation and cortical neuron volume, with consequent increase in brain size and weight (Martinez-Cerdeno et al., 2014). These phenotypes are consistent with the notion that the presence of maternal autoantibodies can affect neuronal development.

STIP1, also known as heat-shock organizing protein (Hop) or STI1, is a co-chaperone that interacts concomitantly with heat-shock proteins 70 and 90 (Hsp70 and HsP90) (Abbas-Terki et al., 2002; Chen et al., 1996; Nicolet and Craig, 1989; Picard, 2002; Smith et al., 1993). The chaperone machinery is thought to provide a buffer for cells to respond to environmental challenges; disturbance of Hsp70/90 chaperone activity decreases cellular resilience to stress (Chen et al., 2015; Hashimoto-Torii et al., 2014; Taipale et al., 2010, 2014). The absence of STIP1 in mice has important consequences for development, including increased apoptosis, DNA damage and death (Beraldo et al., 2013). These phenotypes are rescued by transgenic BAC expression of STIP1 (Beraldo et al., 2013).

In addition to its intracellular role as a co-chaperone, STIP1 is also secreted by a variety of cells (Erlich et al., 2007; Eustace and Jay, 2004; Hajj et al., 2013; Lima et al., 2007; Wang et al., 2010) via extracellular vesicles (Hajj et al., 2013). Extracellular STIP1 can signal via the prion protein (PrP^{C}) to produce a myriad of effects related to brain development (Beraldo et al., 2010, 2013; Caetano et al., 2008; Lopes et al., 2005; Soares et al., 2013). Here, we used *Stip1* heterozygous mice (*STI1*$^{-/+}$ mice), as well as mice overexpresssing four- to fivefold more STIP1 (*STI1*^{TGA} mice), to investigate the consequences of alteration of STIP1 levels *in vivo*.

[redacted]

*Authors for correspondence ([redacted])

Received 30 July 2015; Accepted 4 September 2015

Disease Models & Mechanisms (2015) 8, 1457-1466 doi:10.1242/dmm.022525

TRANSLATIONAL IMPACT

Clinical issue

Autism spectrum disorders (ASD) represent a range of neurodevelopmental disorders with no cure. ASD is characterized by difficulties in communication and socialization, repetitive movements, hyperactivity, impulsivity, and an impaired ability to concentrate and attend to simple tasks. Genetic variance and neurodevelopmental alterations are both thought to contribute to the heterogeneity of the ASD phenotype. Recent studies have demonstrated that some mothers of children with ASD produce antibodies against six specific proteins present in the fetal brain; presumably, these antibodies can interfere with protein function in the developing brain. One of these antibodies targets a protein known as stress inducible phosphoprotein 1 (STIP1). Moreover, a polymorphism for STIP1 was recently identified as a potential risk factor in attention deficit hyperactivity disorder, which shares some phenotypes with ASD. STIP1 is a co-chaperone that mediates the Hsp70/Hsp90 exchange of client proteins. It also triggers prion protein-mediated neuronal signaling.

Results

Here, to investigate the potential involvement of STIP1 in ASD, the authors examine mice that express reduced (50%) or increased (fivefold) levels of STIP1. They show that increased STIP1 levels regulate the abundance of Hsp70 and Hsp90. By contrast, reduced STIP1 levels have no effect on Hsp70, Hsp90 or prion protein levels. Notably, however, mice expressing increased levels of STIP1 show no major phenotype when examined using a range of behavioral tasks, whereas mice expressing reduced levels of STIP1 exhibit attention deficits and are hyperactive.

Implications and future directions

Because attention deficits and hyperactivity are present in ASD, these findings suggest that interference with STIP1 functions (but not increased STIP1 levels) can contribute to ASD-like phenotypes. Changes in STIP1 levels, possibly triggered by the presence of maternal anti-STIP1 antibodies during brain development, might interfere with the development of brain circuits that affect ASD-like behavior. Additional experiments are required to determine whether decreased STIP1 contributes to ASD-like phenotypes by decreasing chaperone capacity in the developing brain, by impairing prion protein signaling, or through some other mechanism, and to define fully the consequences of disturbed STIP1 activity in ASD.

We report that decreased, but not increased, STIP1 levels affect attention and cause hyperactivity in mice, two phenotypes that are related to ASD-like phenotypes. Our results suggest that interference with STIP1 functions, which presumably occur in the presence of STIP1 antibodies, has the potential to contribute to ASD-like phenotypes.

RESULTS

We initially confirmed previous data to show that *STI1*$^{-/+}$ mice present 50% of *STIP1* mRNA levels in their brain, whereas *STI1*^{TGA} mice express almost sixfold more mRNA (Fig. 1A; one-way ANOVA; revealed main effect of genotype $F_{(2.15)}$=8.521, P<0.0001). In contrast, mRNA levels of known STIP1 interaction partners PrP^{C} (Fig. 1B; one-way ANOVA $F_{(2.16)}$=1.475, P=0.2580), Hsp70 (Fig. 1C; one-way ANOVA $F_{(2.16)}$=0.301, P=0.744) and Hsp90 (Fig. 1D; one-way ANOVA $F_{(2.8)}$=1.249, P=0.337) were not altered in the brain of the two lines, compared with control mice.

Protein levels for STIP1 followed mRNA levels for both *STI1*^{TGA} (Fig. 2A; $t_{(15)}$=4.721, P=0.003) and *STI1*$^{-/+}$ (Fig. 2B; $t_{(14)}$=6.433, P<0.0001). PrP^{C} protein levels were not different from controls in both lines (Fig. 2C,D; $t_{(10)}$=1.049, P=0.391 and $t_{(13)}$=1.128, P=0.279, respectively). Interestingly, levels of Hsp70 were decreased by 50% in *STI1*^{TGA} brains (Fig. 2E; $t_{(7)}$=5.846, P=0.0006), whereas no change in Hsp70 levels was detected in *STI1*$^{-/+}$ mice (Fig. 2F; $t_{(7)}$=0.123, P=0.9051), compared with controls. Additionally, Hsp90 levels detected with a pan Hsp90 antibody were doubled in *STI1*^{TGA} brains (Fig. 2G; $t_{(22)}$=4.618, P=0.0001) but not changed in *STI1*$^{-/+}$ brains (Fig. 2H; $t_{(10)}$=0.308, P=0.7639), compared with controls. We then evaluated expression levels of Hsp90α (inducible form) and Hsp90β (constitutive form) in the brains of *STI1*^{TGA} mice and observed that both forms were significantly increased (Fig. 2I,J; $t_{(22)}$=4.618, P=0.0016 and $t_{(16)}$=5.954, P<0.0001, respectively).

Spontaneous locomotor activity in a new environment can provide information on neuropsychiatric phenotypes in mice associated with genetic mutations. The increased number of *Stip1* copies, with concomitant overexpression of Hsp90 and decreased expression of Hsp70 in *STI1*^{TGA} mice did not seem to have any major impact on spontaneous locomotion (Fig. 3A,B; $t_{(29)}$=1.140, P=0.942) or time spent in the center of the box, which provides insight on anxiety-like behavior (Fig. 3C; $t_{(29)}$=1.236, P=0.8669). In contrast, locomotor activity and total locomotion in a new environment were increased in *STI1*$^{-/+}$ mice (Fig. 3D,E; $t_{(44)}$=1.879, P=0.0078). However, *STI1*$^{-/+}$ mice did not show increased anxiety-like behavior, as determined by the time spent in the center of the box (Fig. 3F; $t_{(40)}$=1.221, P=0.341). We also examined another cohort of *STI1*$^{-/+}$ mice using automated metabolic cages. In this experiment, which mimics the home cage environment, *STI1*$^{-/+}$ mice again showed hyperactivity during the day and night periods, considering both total activity (Fig. 3G; $t_{(14)}$=2.558, P=0.0228 and $t_{(14)}$=2.230, P=0.0426) and ambulatory activity (Fig. 3H; $t_{(14)}$=2.420, P=0.00297 and $t_{(14)}$=2.230, P=0.0426). Given this increased motor activity, *STI1*$^{-/+}$ mice also demonstrated less sleep time (periods of inactivity) (Fig. 3I; $t_{(14)}$=3949, P=0.0015 and $t_{(14)}$=2.724, P=0.0165). Also, *STI1*$^{-/+}$ mice showed increased consumption of O_2 during the light and dark cycle (Fig. 3J; $t_{(14)}$=2.464, P=0.027 and $t_{(14)}$=2.169, P=0.047) and CO_2 production during the dark cycle, but not in the light cycle (Fig. 3K; $t_{(14)}$=2.307, P=0.036 and $t_{(14)}$=1.360, P=0.195). No differences were observed in other parameters such as respiratory ratio (Fig. 3L; $t_{(14)}$=0.4455, P=0.6627 and $t_{(14)}$=0.459, P=0.653), food consumption (Fig. 3M; $t_{(14)}$=0.5216, P=0.6101 and $t_{(14)}$=0.6134, P=0.5494), water consumption (Fig. 3N; $t_{(14)}$=1.801, P=0.0933 and $t_{(14)}$=0.2752, P=0.7872), and heat production (Fig. 3O; $t_{(14)}$=1.014, P=0.3276 and $t_{(14)}$=0.1935, P=0.8494) comparing *STI1*$^{-/+}$ to *STI1*$^{+/+}$ mice for both cycles (light and dark).

In order to test for other neuropsychiatric-like behaviors as a result of altered STIP1 levels we tested both *STI*^{TGA} and *STI1*$^{-/+}$ mice for anxiety-like behavior (Fig. 4A-D) and depression-like behavior (Fig. 4E,F). Given the hyperactivity of *STI1*$^{-/+}$ mice, we also decided to investigate whether they had alterations in compulsive-like behavior, assessed by measurement of self-grooming and marble burying (Fig. 4G-I). There was no difference in the behavior of either *STI*^{TGA} (Fig. 4A,B,E) or *STI1*$^{-/+}$ (Fig. 4C,D,F-I) mice compared with control mice in all these behavioral tasks: time spent in the open arm (Fig. 4C; t_{19}=0.310, P=0.7590), time spent in the closed arm (Fig. 4D; $t_{(19)}$=0.3730, P=0.7133), forced swim test (Fig. 4F; $t_{(12)}$=1.184, P=0.2594), grooming bouts (Fig. 4H; $t_{(20)}$=0.7848, P=0.4418), time grooming (Fig. 4G; $t_{(20)}$=0.6072, P=0.5505) and marble burying (Fig. 4I; $t_{(21)}$=0.4956, P=0.6253).

Disease Models & Mechanisms (2015) 8, 1457-1466 doi:10.1242/dmm.022525

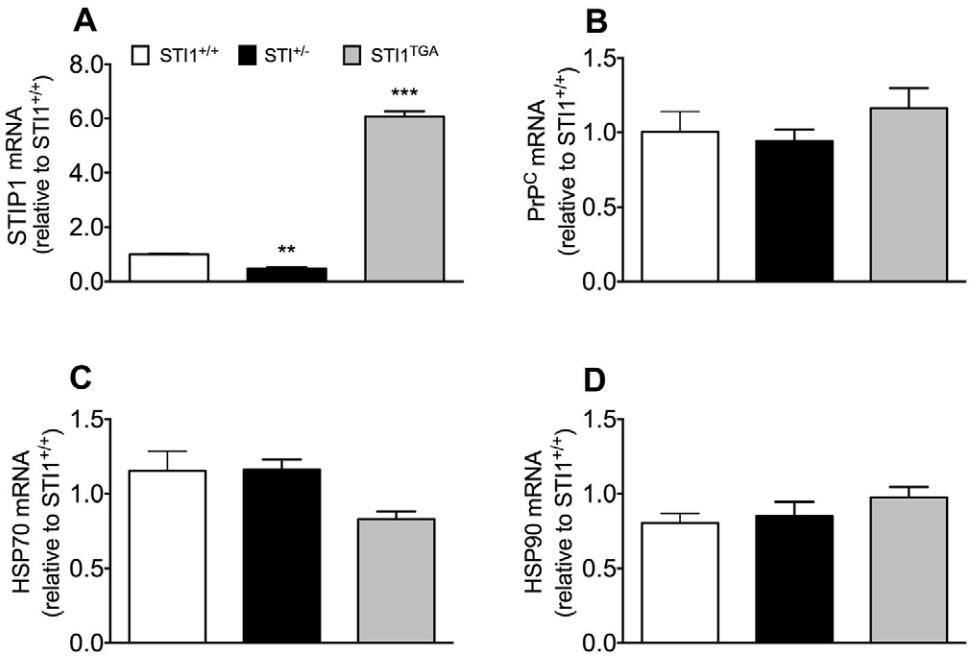


Fig. 1. Analyses of mRNA for STIP1 partners in *STI1*$^{+/+}$, *STI1*$^{-/+}$ and *STI1*^{TGA} mouse brains. (A) *STIP1* mRNA expression (n=9 *STI1*$^{+/+}$, n=5 *STI1*^{TGA} and n=4 *STI1*$^{-/+}$). (B) *PrP*^{C} mRNA expression (n=8 *STI1*$^{+/+}$, n=4 *STI1*^{TGA} and n=7 *STI1*$^{-/+}$). (C) *Hsp70* mRNA expression (n=8 *STI1*$^{+/+}$, n=4 *STI1*^{TGA} and n=7 *STI1*$^{-/+}$). (D) *Hsp90* mRNA expression (n=3 *STI1*$^{+/+}$, n=4 *STI1*^{TGA} and n=4 *STI1*$^{-/+}$). Results are presented as means±s.e.m.; data were analyzed and compared by one-way ANOVA and Bonferroni multiple comparisons post-hoc test; **P<0.001 and ***P<0.0001 compared with control.

Next, we investigated spatial navigation memory in both *Stip1* mutant mice using the Morris water maze (MWM). Neither *STI1*^{TGA} nor *STI1*$^{-/+}$ mice presented deficits in acquisition or retrieval of spatial memory in the MWM. For both *STI1*^{TGA} and *STI1*$^{-/+}$, performance during the 4-day acquisition phase was indistinguishable from their wild-type controls in terms of latency to find the target (Fig. 5A; RM-ANOVA $F_{(1,13)}$=0.062, P=0.806) or speed (Fig. 5C; $F_{(1,10)}$=0.215, P=0.652). When spatial memory retrieval was performed on the day-5 probe trial, again no differences were observed between *STI1*^{TGA} and *STI1*$^{-/+}$ mice, compared with their wild-type controls, for time spent investigating the target quadrant (Fig. 5D; $F_{(1,13)}$=1.046, P=0.3251) or latency (Fig. 5E; $F_{(1,10)}$=0.215, P=0.294).

Given the hyperactivity phenotype and genetic data suggesting the potential of STIP1 to be linked to ADHD (Mick et al., 2011), a trait commonly found in ASD (Gadow et al., 2006; Goldstein and Schwebach, 2004; Lee and Ousley, 2006; Mulligan et al., 2009), we also determined whether changes in STIP1 levels affected attentional processing. For this, we used the 5-CSRTT. After mice were trained to perform to a criterion (>80% accuracy, <20% omissions) at a 2 s stimulus duration, we assessed attentional performance by using reduced stimulus durations in probe trials (1.5, 1, 0.8 and 0.6 s stimulus durations) as previously described (Romberg et al., 2011). We observed no differences in attentional performance in *STI1*^{TGA} mice compared with their littermate controls. There was no difference in accuracy (Fig. 6A; RM-ANOVA showed no effect of genotype $F_{(1,20)}$=0.0057, P=0.9403, main effect of stimulus duration $F_{(3,60)}$=12.14, P<0.0001 and no significant interaction $F_{(3,60)}$=0.1328, P=0.9402) or omission rates (Fig. 6B; RM-ANOVA showed no effect of genotype $F_{(1,18)}$=0.2429, P=0.6281, main effect of stimulus duration $F_{(3,54)}$=17.62, P<0.0001 and significant interaction $F_{(3,54)}$=3.854, P=0.0143). Post-hoc analysis showed that there was no significant difference between *STI1*^{TGA} mice and controls. There was also no difference in premature responses, a measure of impulsivity (Fig. 6C; RM-ANOVA showed no effect of genotype $F_{(1,9)}$=0.00056, P=0.9419, no effect of stimulus duration $F_{(3.27)}$=0.8254, P=0.4914 and no significant interaction $F_{(3,27)}$=1.109, P=0.3625). Moreover, we did not find any difference in motivation, measured as latency to touch the screen (Fig. 6D; RM-ANOVA showed no effect of genotype $F_{(1,9)}$=3.399, P=0.0983, main effect of stimulus duration $F_{(3.30)}$=4.281, P=0.0125 and no significant interaction $F_{(3,30)}$=2.332, P=0.0941). Compulsivity and motivation were not altered either, as assessed by perseverative responses (Fig. 6F; RM-ANOVA, showed no effect of genotype $F_{(1,9)}$=3.974, P=0.0774, main effect of stimulus duration $F_{(3,27)}$=4.808, P=0.0083 and no significant interaction $F_{(3,27)}$=0.1773, P=0.9108) and reward collection latency (Fig. 6E; RM-ANOVA showed no effect of genotype $F_{(1,10)}$=1.291, P=0.2824, no effect of stimulus duration $F_{(3,30)}$=2.162, P=0.1132 and no significant interaction $F_{(3.30)}$=0.7372, P=0.5381).

In contrast, when attentional demand was increased, *STI1*$^{-/+}$ mice presented decreased accuracy (Fig. 6G; RM-ANOVA, main effect of genotype $F_{(1,25)}$=6.872, P=0.0147, main effect of stimulus duration $F_{(3,75)}$=41.95, P<0.0001, significant interaction effect $F_{(3,75)}$=4.170, P=0.0087) and increased omission rates (Fig. 6H; RM-ANOVA, main effect of genotype $F_{(1,25)}$=6.584, P=0.0167, main effect of stimulus duration $F_{(3,75)}$=24.62, P<0.0001, significant interaction effect $F_{(3,75)}$=3.401, P=0.0220). Post-hoc analysis revealed that the *STI1*$^{-/+}$ mice were significantly impaired in both accuracy and omissions at the 0.6 s stimulus duration. The worse performance of *STI1*$^{-/+}$ mice was not related to changes in motivation (latency to respond to the stimulus, RM-ANOVA, no effect of genotype $F_{(1,25)}$=0.01856, P=0.8925, no effect of stimulus duration $F_{(3,75)}$=1.720, P=0.1702, no interaction $F_{(3,75)}$=1.070, P=0.3669). There was also no difference in latency to retrieve the reward following a correct response (RM-ANOVA, no effect of genotype $F_{(1,25)}$=0.03176, P=0.8600, no effect of stimulus duration $F_{(3,75)}$=0.3997, P=0.7536, no interaction $F_{(3,75)}$=1.785, P=0.8284). Moreover, we detected no increase in premature responses (RM-ANOVA, no effect of genotype $F_{(1,25)}$=0.0958, P=0.7595, main effect of stimulus duration $F_{(3,75)}$=2.907, P=0.0401, no interaction effect $F_{(3,75)}$=2.017, P=0.1187) or perseverative responses (RM-ANOVA, no effect of genotype $F_{(1,25)}$=0.04188, P=0.8395, main effect of stimulus duration $F_{(3,75)}$=6.975, P=0.0003, no interaction effect $F_{(3,75)}$=1.139, P=0.3389).

Disease Models & Mechanisms (2015) 8, 1457-1466 doi:10.1242/dmm.022525

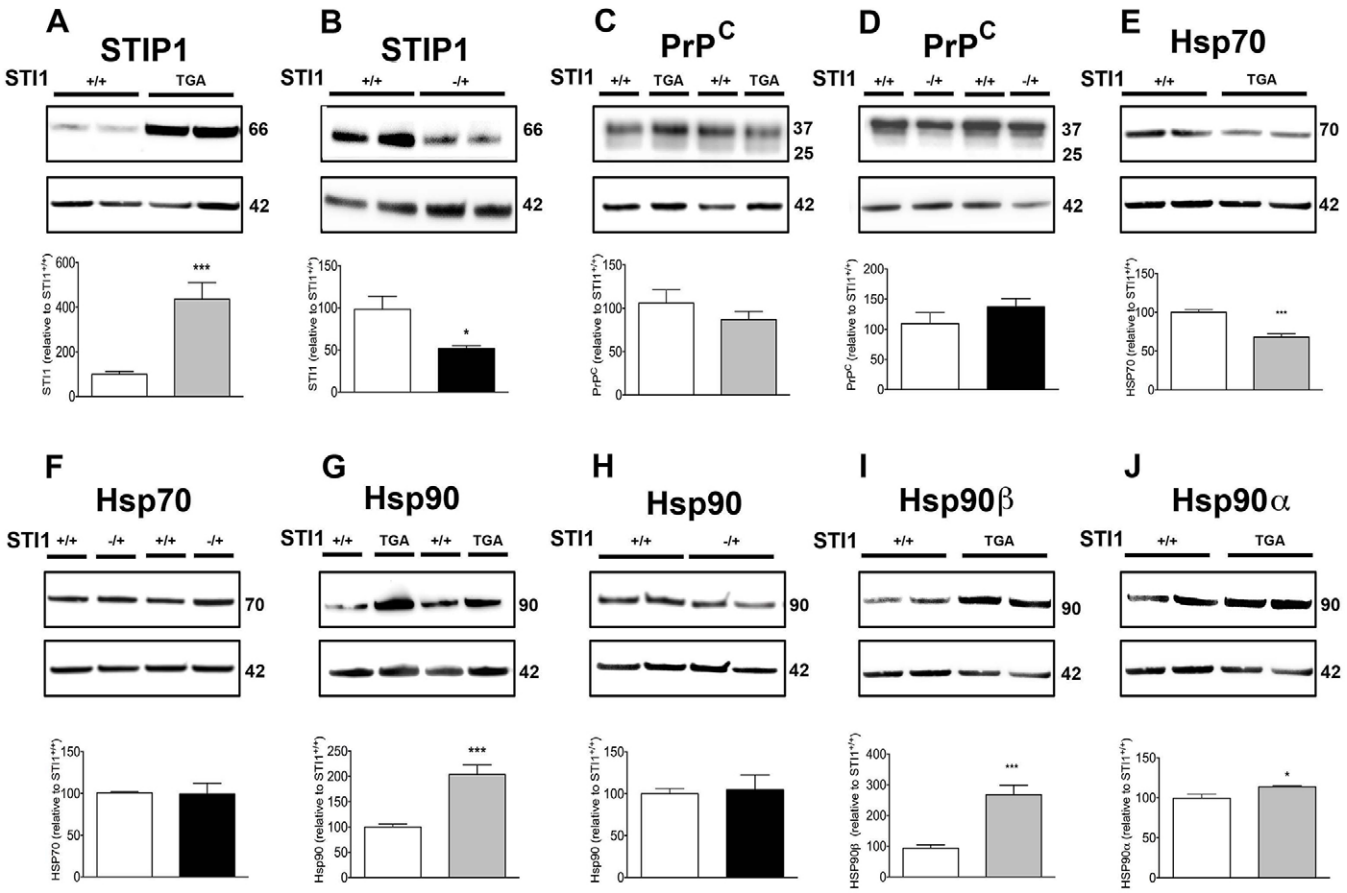


Fig. 2. Analyses of protein levels for STIP1 partners in *STI1*$^{+/+}$, *STI*$^{-/+}$ and *STI1*^{TGA} mouse brains. (A,B) STIP1 expression in *STI1*^{TGA} (n=9 *STI1*$^{+/+}$ and n=8 *STI1*^{TGA}) and *STI1*$^{-/+}$ mice (n=8 *STI1*$^{+/+}$ and n=8 *STI1*$^{-/+}$). (C,D) PrP^{C} expression in *STI1*^{TGA} (n=6 *STI1*$^{+/+}$ and n=6 *STI1*^{TGA}) and *STI1*$^{-/+}$ mice (n=6 *STI1*$^{+/+}$ and n=9 *STI1*$^{-/+}$). (E,F) Hsp70 expression in *STI1*^{TGA} (n=5 *STI1*$^{+/+}$ and n=4 *STI1*^{TGA}) and *STI1*$^{-/+}$ mice (n=5 *STI1*$^{+/+}$ and n=4 *STI1*$^{-/+}$). (G,H) HSP90 expression in *STI1*^{TGA} (n=10 *STI1*$^{+/+}$ and n=14 *STI1*^{TGA}) and *STI1*$^{-/+}$ mice (n=6 *STI1*$^{+/+}$ and n=6 *STI1*$^{-/+}$). (I,J) Hsp90β (n=10 *STI1*$^{+/+}$ and n=8 *STI1*^{TGA}) and Hsp90α (n=5 *STI1*$^{+/+}$ and n=4 *STI1*^{TGA}) in *STI1*^{TGA} mice. Results are presented as means±s.e.m.; data were analyzed and compared by Student's t-test; *P<0.05 and ***P<0.0001 compared with control.

DISCUSSION

The present experiments tested whether alterations in STIP1 levels have consequences for psychiatric-like behaviors in mice. Our results suggest that decreased, but not increased, STIP1 levels cause significant behavioral alterations in mice. Spatial learning and memory, as well as anxiety and depression-like behavior do not seem to be affected by reduced STIP1 levels. However, mutant mice deficient for STIP1 are hyperactive and present attention deficits.

STIP1 has recently emerged as a protein of potential interest in ASD and endophenotypes related to ASD. Maternal autoantibodies against STIP1 have been identified in mothers of children with ASD (Braunschweig et al., 2013). Moreover, recent global-wide association study (GWAS) analysis identified a polymorphism in *STIP1* (the human gene coding for STIP1/HOP) as a potential risk factor in a population of individuals diagnosed with attention-deficit disorder (Mick et al., 2011), a co-morbidity often associated with ASD (Brimberg et al., 2013; Goldani et al., 2014). The consequences of this polymorphism for STIP1 expression is unknown, but the presence of autoantibodies against STIP1 might affect expression levels of the protein, given that antibodies can penetrate the blood brain barrier of the fetus during pregnancy (Braunschweig et al., 2012a; Diamond et al., 2009; Fox et al., 2012; Zhang et al., 2012). Indeed, maternal antibodies that recognize STIP1 and other targets when injected in pregnant rodents or developing pups can lead to offspring with abnormal neurons and behaviors that relate to ASD (Braunschweig et al., 2012b; Camacho et al., 2014). To a degree, *STI1*$^{-/+}$ mice model this early developmental deficit in STIP1 levels. However, in *STI1*$^{-/+}$ mice STIP1 expression is persistently decreased through life, which could also have important consequences for the phenotypes described.

STIP1 is a modular protein containing several tetratricopeptide (TRP) repeat domains and aspartate-proline (DP) reach domains (Taipale et al., 2010). TRP1 and TRP2B can interact with Hsp70 (Flom et al., 2007; Scheufler et al., 2000), whereas TPR2A is required for interaction with Hsp90 (Flom et al., 2007, 2006). Hsp90 activity is regulated by STIP1 and previous work has shown that in mice no other co-chaperone can replace STIP1 (Beraldo et al., 2013). Recent experiments have indicated that the chaperone machinery, activated by the transcription factor heat shock factor 1 (HSF1), is responsible for preventing damaging effects from environmental factors in the developing brain (Hashimoto-Torii et al., 2014). Indeed, the chaperone machinery can buffer many stresses at the cellular level and, therefore, it is not surprising that functional changes in its components have physiological consequences.

In addition to its intracellular chaperone function, STIP1 is also secreted by a myriad of cells, including astrocytes via an extracellular vesicle population, which includes exosomes (Hajj et al., 2013). Extracellular STIP1 also mediates important physiological responses in the brain. Acting as a trophic factor to engage PrP^{C} to signal in neurons, it regulates neuritogenesis and

Disease Models & Mechanisms (2015) 8, 1457-1466 doi:10.1242/dmm.022525

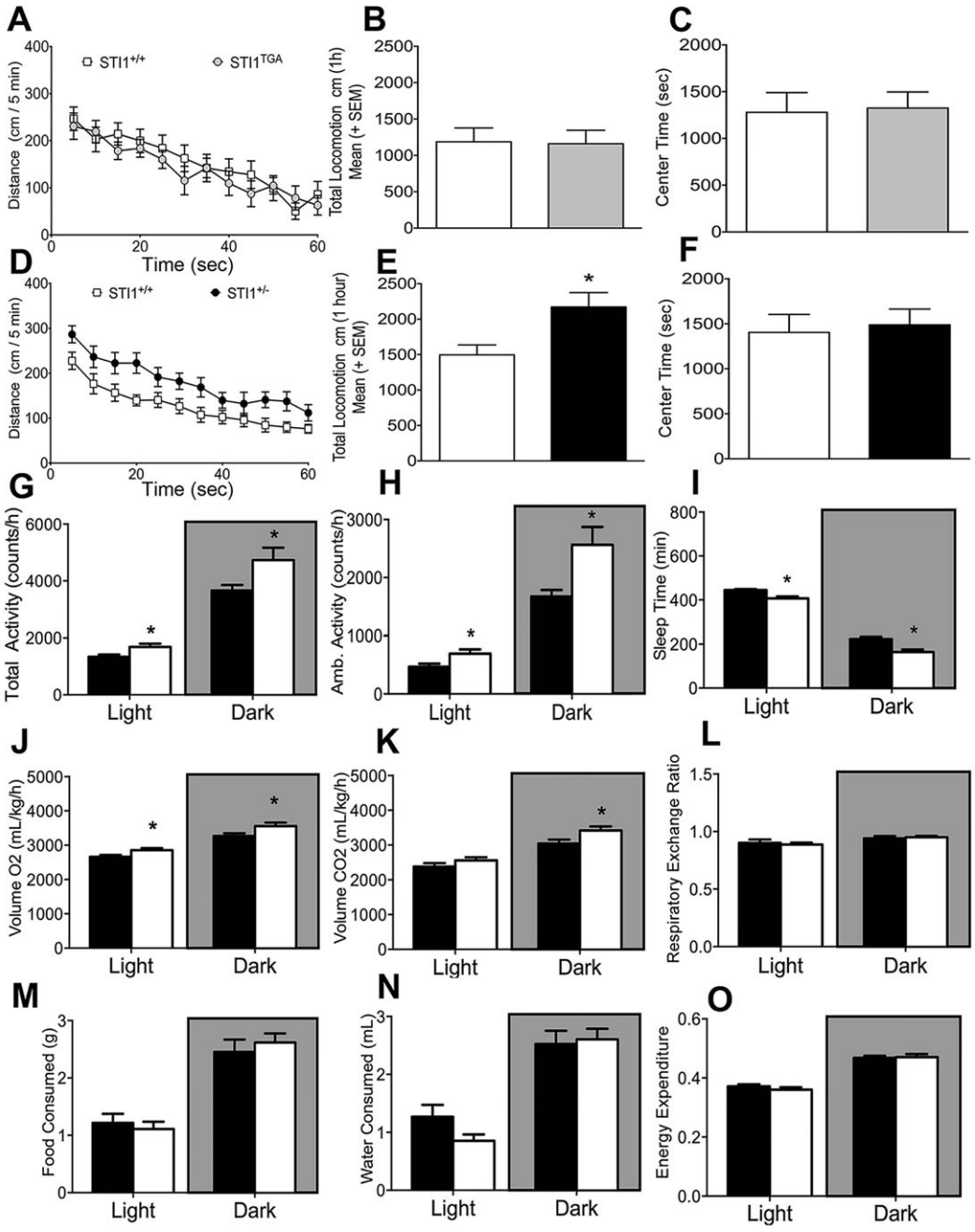


Fig. 3. Locomotor activity in *STI1*^{TGA} and *STI1*$^{-/+}$ mice and metabolic analyses in *STI1*$^{-/+}$ mice. (A) Horizontal locomotor activity in an open-field for *STI1*^{TGA} (n=14) and *STI*$^{+/+}$ control mice (n=14). (B) Cumulative 1 h locomotion for *STI1*^{TGA} (n=14) and *STI*$^{+/+}$ control mice (n=14). (C) Time spent in the center of the locomotion boxes for *STI1*^{TGA} (n=14) and *STI*$^{+/+}$ control mice (n=14). (D) Horizontal locomotor activity in an open-field for *STI1*$^{-/+}$ (n=8) and *STI*$^{+/+}$ control mice (n=8). (E) Cumulative 1 h locomotion for *STI1*$^{-/+}$ (n=22) and *STI*$^{+/+}$ control mice (n=24). (F) Time spent in the center of the locomotion boxes for *STI1*$^{-/+}$ (n=22) and *STI*$^{+/+}$ control mice (n=24). (G) Total activity in metabolic cages for *STI1*$^{-/+}$ (n=8) and *STI*$^{+/+}$ control mice (n=8). (H) Ambulatory activity in metabolic cages for *STI1*$^{-/+}$ (n=8) and *STI*$^{+/+}$ control mice (n=8). (I) Sleep time for *STI1*$^{-/+}$ (n=8) and *STI*$^{+/+}$ control mice (n=8). (J) VO_2 for *STI1*$^{-/+}$ (n=8) and *STI*$^{+/+}$ control mice (n=8). (K) VCO_2 for *STI1*$^{-/+}$ (n=8) and *STI*$^{+/+}$ control mice (n=8). (L) Respiratory exchange ratio for *STI1*$^{-/+}$ (n=8) and *STI*$^{+/+}$ control mice (n=8). (M) Food consumption for *STI1*$^{-/+}$ (n=8) and *STI*$^{+/+}$ control mice (n=8). (N) Water consumption for *STI1*$^{-/+}$ (n=8) and *STI*$^{+/+}$ control mice (n=8). (O) Energy expenditure for *STI1*$^{-/+}$ (n=8) and *STI*$^{+/+}$ control mice (n=8). Results are presented as means±s.e.m.; data were analyzed and compared by Student's t-test; *P<0.05 compared with control.

neuronal survival (Beraldo et al., 2010; Lopes et al., 2005; Roffe et al., 2010). STIP1 has a role in functional recovery in stroke (Beraldo et al., 2013; Lee et al., 2013). Moreover, STIP1 also modulates toxicity of Aβ peptides in models of Alzheimer's disease (Brehme et al., 2014; Ostapchenko et al., 2013).

It is remarkable that mice with increased levels of STIP1 (up to almost fivefold) do not present any major behavioral alteration. In the extensive evaluation of cognitive phenotypes in this study, which included anxiety and depression-like behaviors, spatial memory and attention, we showed that *STI1*^{TGA} mice perform as well as littermate controls. These results suggest that strategies to increase STIP1 levels should not cause toxicity with consequences for brain functions. This is important, given that increased STIP1 levels might be protective against insults such as stroke-mediated

Disease Models & Mechanisms (2015) 8, 1457-1466 doi:10.1242/dmm.022525

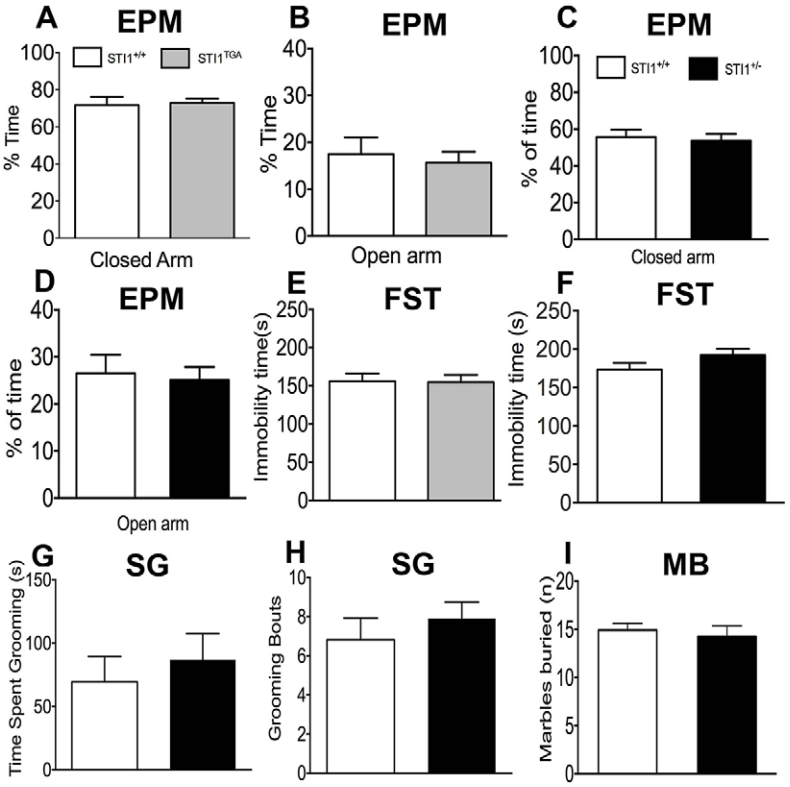


Fig. 4. Anxiety-like behavior, depression-like behavior, social behavior and compulsivity in *STI1*^{TGA} and *STI1*$^{-/+}$ mice. (A) Percentage of time spent in the closed arm for *STI1*^{TGA} (*n*=17) and control mice (*n*=14). (B) Percentage of time spent in the open arm for *STI1*^{TGA} (*n*=17) and control mice (*n*=14). (C) Percentage of time spent in the closed arm for *STI1*$^{-/+}$ (*n*=13) and control mice (*n*=10). (D) Percentage of time spent in the open arm for *STI1*$^{-/+}$ (*n*=13) and control mice (*n*=10). (E) Immobility time in the forced-swimming test for *STI1*^{TGA} (*n*=17) and control mice (*n*=14). (F) Immobility time in the forced-swimming test for *STI1*$^{-/+}$ (*n*=6) and control mice (*n*=8). (G,H) Time spent grooming and number of grooming bouts for *STI1*$^{-/+}$ (*n*=11) and control mice (*n*=11). (I) Marbles buried by *STI1*$^{-/+}$ (*n*=12) and control mice (*n*=12).

cell death and in Alzheimer's disease (Beraldo et al., 2013; Ostapchenko et al., 2013). Interestingly, whereas increased levels of STIP1 seem to affect the chaperone machinery, prion protein expression is not affected by decreasing the level of Hsp70 and increasing Hsp90. These consequences of increased STIP1 seem to occur at the post-translational level, given that mRNAs for Hsp70 and 90 were not affected. It is unknown at the moment whether increased STIP1 levels stabilize a complex containing Hsp90, preferentially leading to increased turnover of Hsp70.

At present, the exact mechanism by which decreased STIP1 levels affect psychiatric-like behavior is still unknown. Although it is possible that decreased levels of STIP1 during early development have persistent effects in brain circuits, culminating with hyperactivity and attentional deficits, we cannot discard the possibility that STIP1 plays a role in regulating circuitry function in the adult brain. Our experiments at the moment do not discriminate whether the phenotypes observed in mutant mice result from decreased STIP1 co-chaperone function, diminished STIP1 extracellular signaling or both. Our results suggest that reduced levels of STIP1 have important consequences for behavior and seem to affect brain circuits that regulate attention. It is possible that exposure to STIP1 antibodies during pregnancy could reduce STIP1 levels, which, based on the present results, would have important consequences. Future experiments are required to define potential mechanisms as well as the consequences of disturbed STIP1 activity in ASD.

MATERIALS AND METHODS

Animals

STI1$^{-/+}$ and *STI1*^{TGA} mice were generated as described (Beraldo et al., 2013). Both mouse lines were in the C57BL/6J background. All experimental procedures were conducted in compliance with the Canadian Council of Animal Care guidelines for use and care of animals and in accordance with approved animal use protocols at the University of Western Ontario (2008/127). Animals were housed in groups of two or four per cage. Mice were kept in a temperature-controlled room with a 12/12 light/dark cycle (7 am/7 pm) with food and water provided *ad libitum* unless stated otherwise. For behavioral studies, only male mice were used. Mice were randomized and the experimenter was blind to genotypes. For most of the behavioral tasks, software-based analyses were used to score mice performance with minimum human interference.

qPCR and Western blot

For real-time quantitative PCR (qPCR), brain tissues were homogenized in Trizol and total RNA was extracted using the Aurum Total RNA kit for fatty and fibrous tissue (Bio-Rad, Hercules, CA, USA). qPCR were performed as previously described (Martins-Silva et al., 2011). Primer sequences: STIP1-F, 5′-GCCAAGAAAGGAGACTACCAG-3′; STIP1-R, 5′-TCATAGGTTCGTTTGGCTTCC-3′; HsP90-F, 5′-CCACCCTGCTCTGTACTACT-3′; HsP90-R, 5′-CCAGGGCATCTGAAGCATTA-3′; HsP70-R, 5′-ACCTTGACAGTAATCGGTGC-3′; HsP70-F, 5′-CTCCCGGTGTGGTCTAGAAA-3′; PRP-F, 5′-GAACCATTTCAACCGAGCTG-3′; PRP-R, 5′-CATAGTCACAAAGAGGGCCAG-3′; Actin-F, 5′-TGGAATCCTGTGGCATCCATGA-3′; and Actin-R, 5′-AATGCCTGGGTACATGGTGGTA-3′. Immunoblot analysis was carried out as described previously (Beraldo et al., 2013). The antibodies used were anti-STIP1 (1:5000, in-house antibody generated by Bethyl Laboratories Montgomery, USA using recombinant STIP1) (Beraldo et al., 2013), anti-Hsp90 (1:1000), anti-Hsp70 (1:1000), anti-Hsp90α (1:1000), anti Hsp90β (1:1000) (Cell Signaling, Danvers, USA) and anti-PrP 8H4 (1:2000) (Abcam, Cambrige, UK).

Locomotor activity

Mice were acclimated to the testing room for 30 min prior to beginning the test; locomotor activity was automatically recorded (Omnitech Electronics Inc., Columbus, USA). Mice were placed in the center of the apparatus and locomotor activity was measured at 5 min intervals for 1 h as described previously (Martyn et al., 2012).

Elevated plus maze

To access anxiety-like behavior, mice were acclimated to the testing room for 30 min prior to beginning the test and then placed in the center of the

Disease Models & Mechanisms (2015) 8, 1457-1466 doi:10.1242/dmm.022525

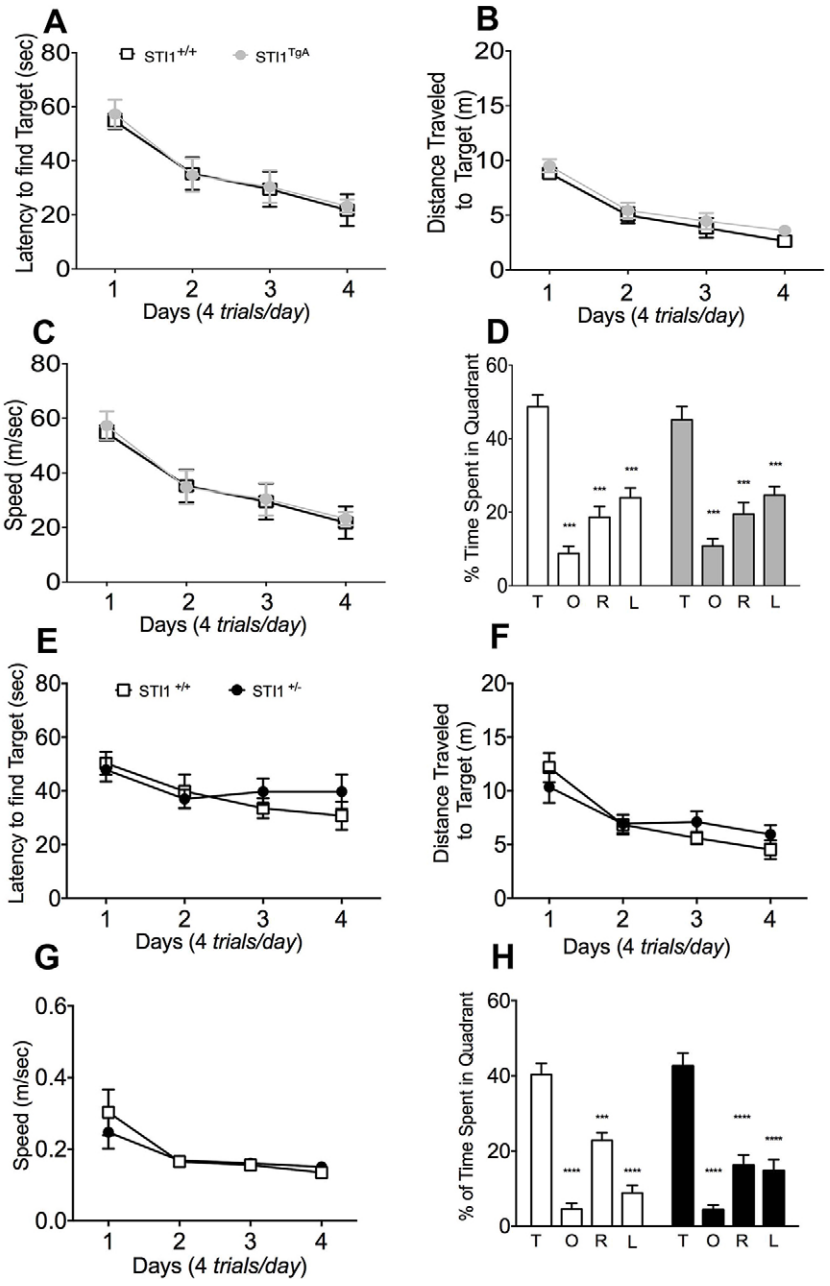


Fig. 5. Spatial memory in *STI1*^{TGA} and *STI1*$^{-/+}$ mice. For the tests, n=14 *STI1*$^{+/+}$ and 14 *STI1*^{TGA} mice were used to test spatial memory in *STI1*^{TGA} mice and n=11 *STI1*$^{+/+}$ and 11 *STI1*$^{-/+}$ for *STI1*$^{-/+}$ mice. (A) Latency to find the platform. (B) Distance traveled. (C) Speed for *STI1*^{TGA} mice. (D) Percentage time spent by *STI1*^{TGA} mice and controls in target quadrant (T) and in opposite (O), right (R) and left (L) quadrants was measured on day 5 in a 60 s probe trial with the platform removed. (E) Latency to find the platform. (F) Distance traveled. (G) Speed for *STI1*$^{-/+}$ mice. (H) Percentage time spent by *STI1*$^{-/+}$ mice and controls in each quadrant was measured on day 5 in a 60 s probe trial with the platform removed. Results are presented as means±s.e.m.; data were analyzed and compared by two-way ANOVA; ***P<0.001 and ****P<0.0001 compared with time spent in target quadrant.

elevated plus maze (Med Associates Inc., St Albans, USA). The activity was recorded and videos were analyzed using ANY-maze software (Stoelting Co., USA) to determine the amount of time spent in the closed and open sections of the maze.

Forced swimming test

Depressive-like behavior was assessed by a forced swim test (FST) as described previously (Martyn et al., 2012). Briefly, mice were placed in a 2 l beaker containing 1.7 l of water at 25-27°C for 6 min. Experimental sessions were recorded and immobility time was evaluated using ANY-Maze Software (Stoelting Co., USA). Data obtained from the last 4 min of testing were used for the analysis.

Morris water maze

The spatial version of Morris water maze (MWM) was conducted as described previously (Kolisnyk et al., 2013; Martyn et al., 2012; Vorhees and Williams, 2006). Briefly, the task was performed in a 1.5-m diameter/1-m deep pool filled with water at 25°C. Spatial cues, 40×40 cm boards containing black symbols (vertical and horizontal stripes, triangles, squares and circles), were placed on the walls distributed around the pool and the platform was submerged 1 cm below the surface of the water. Mice were submitted to four training trials a day (90 s each) for four consecutive days with a 15 min intertrial interval. On day 5, memory was assessed by a single 60 s trial on which the platform was removed and the time spent in the target quadrant was evaluated. All the experimental sessions were recorded and analyzed using the ANY-Maze Software.

Five-choice serial reaction time task

The five-choice serial reaction time task (5-CSRTT) was used to evaluate attention in mice as described previously (Kolisnyk et al., 2013; Romberg et al., 2011). Mice were trained in the 5-CSRTT in automated Bussey–SaksidaTouch screen systems (Campden Instruments Limited, Loughborough, EN) and the data collected using ABET II Touch software V.2.18 (Lafayette Instruments, Lafayette, USA). Mice were submitted to a pre-training program, which consisted of first habituating the mouse to the testing chamber with the lights off for 10 min. The next day, the mouse was

Disease Models & Mechanisms (2015) 8, 1457-1466 doi:10.1242/dmm.022525

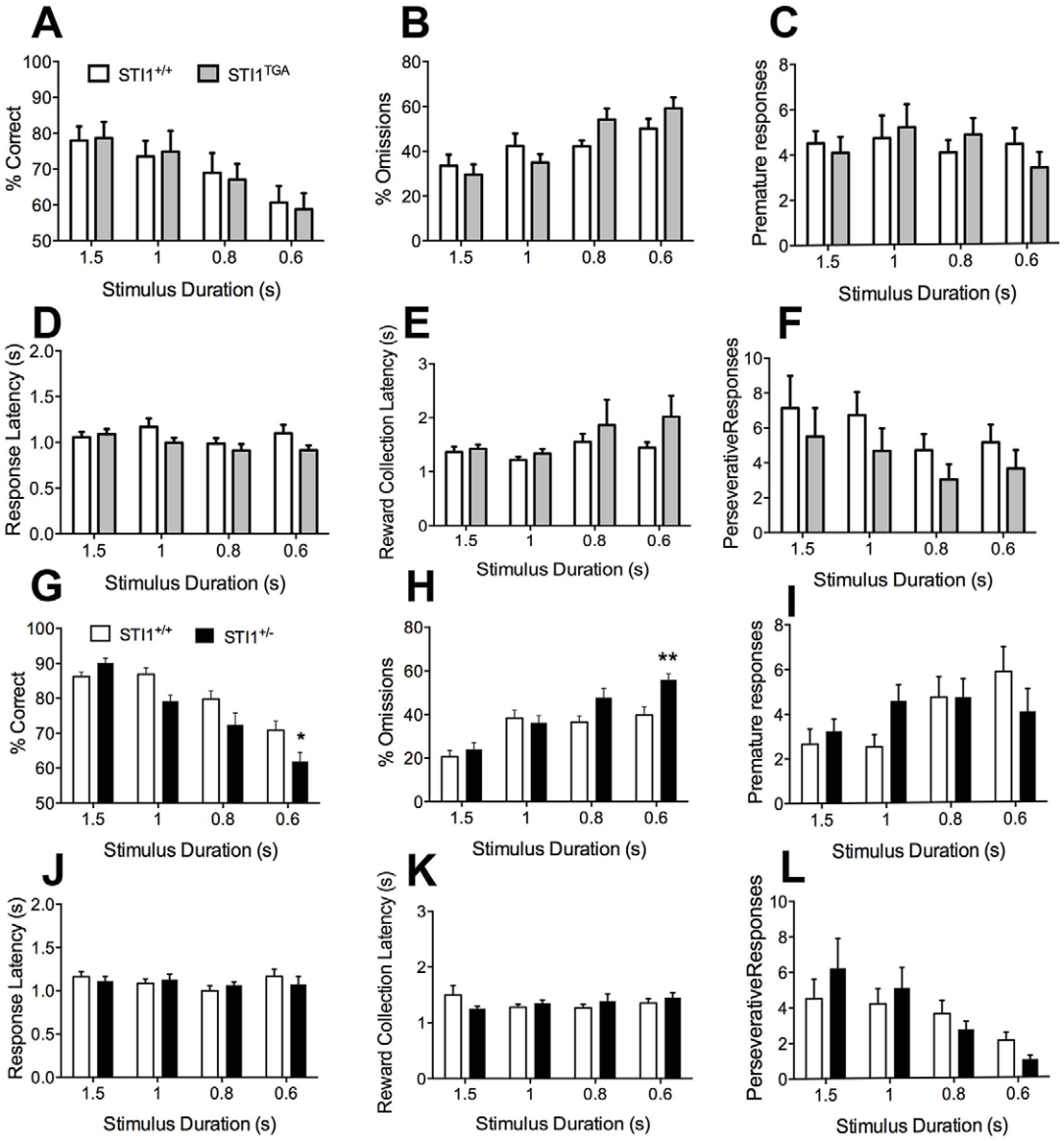


Fig. 6. Five-choice serial reaction time task used to measure attention in *STI1*^{TGA} and *STI1*$^{-/+}$. For the tests, n=10 *STI1*$^{+/+}$ and 10 *STI1*^{TGA} mice were used to test attention in *STI1*^{TGA} mice and n=13 *STI1*$^{+/+}$ and 13 *STI1*$^{-/+}$ for *STI1*$^{-/+}$ mice. (A) Accuracy during probe trial sessions. (B) Rate of omission. (C) Premature responses. (D) Response latency. (E) Reward collection latency. (F) Perseverative responses for *STI1*^{TGA} mice. (G) Accuracy during probe trial sessions. (H) Rate of omission. (I) Premature responses. (J) Response latency. (K) Reward collection latency. (L) Perseverative response for *STI1*$^{-/+}$ mice. Results are presented as means±s.e.m.; data were analyzed and compared by RM-ANOVA; *P<0.05, **P<0.001 compared with control.

put in the chamber with the lights off for 20 min. After two days of habituation with no reward been offered, the reward tray was primed with 11% fat strawberry milkshake (Nielson - Saputo Dairy Products) and a tone was played when the mouse entered the reward tray. This was repeated for the next 2 days for 40 min sessions. Whenever the mouse returned to the reward tray, the reward was offered and paired with a tone (phase I). The following training phase consisted in pairing the reward with the presentation of a random stimulus (flash of light in one of the five windows), which is removed after 30 s. At this phase, if the mouse touched the screen when the stimulus was displayed, it received a reward. This cycle was repeated until the mouse completed 30 trials or 60 min timeout (phase II). At phase III of the training, the stimulus was displayed randomly in one of the five windows. The mouse had to touch the window where the stimulus was displayed to receive the reward paired with a tone. Similar to phase II, this cycle was repeated until the mouse completed 30 trials or 60 min timeout. The next step (phase IV) was identical to phase III except by the fact that the mouse had to poke its nose into the reward trail to initiate the task. This process was repeated in the last phase of the pre-training (phase V); however, if the mouse touched an incorrect screen, it received a 5 s timeout and the light in the chamber was turned on. After the mouse had finished pre-training and reached criterion at 4 s and 2 s stimulus duration (80% accuracy, 20% omission for three consecutive days), mice were probed for attention deficits following probe trial schedules: each mouse was tested over two sessions at 1.5, 1.0, 0.8 and 0.6 s stimulus duration (the order of the probe trial sessions was randomized and the groups counterbalanced). Between each different stimulus duration, each mouse was returned to a 2 s stimulus for two consecutive sessions. Number of trials to criterion, accuracy, omission, reward collection latency and perseverative response were analyzed.

Metabolic assessments

Oxygen consumption, carbon dioxide production, respiratory exchange ratio (RER), carbon dioxide production, water and food intake and physical activity were simultaneously measured for adult *STI1*$^{+/+}$ and *STI1*$^{+/-}$ mice by using the Comprehensive Lab Animal Monitoring System (CLAMS) interfaced with Oxymax Software (Columbus Instruments, Columbus, OH, USA) as previously described in detail (Guzman et al., 2013; Kolisnyk et al.,

2013). Briefly, mice were individually housed in the metabolic chambers with *ad libitum* access to water and food. Following a 16-h habituation period, all measurements were obtained every 10 min for 24 h (12 h light/12 h dark).

Marble burying task

A marble burying task was used to assess repetitive and anxiety-like behavior as previously described (Deacon, 2006).

Assessment of self-grooming

Self-grooming was assessed to evaluate repetitive behavior, as previously described (McFarlane et al., 2008). Briefly, each mouse was placed individually in a clean, empty, cage and given a 10 min habituation period, after which the mice were filmed for another 10 min. Cumulative time spent grooming and number of grooming bouts were counted by an experimenter blinded to the genotypes of the mice.

Statistical analyses

Data are presented as mean±s.e.m. Statistical analyses were performed using SigmaStat 3.5 software. Student's *t*-test was used to compare two experimental groups and for comparison of several experimental groups, two-way ANOVA or two-way repeated-measures ANOVA were used as required. Tukey's post hoc comparison was used when required.

Competing interests

The authors declare no competing or financial interests.

Author contributions

F.H.B., M.A.M.P., V.F.P., and R.G. conceived and designed experiments. F.H.B., A.T., B.K., P.H.H., R.G., X.D.J., A.C.M., J.F., D.F.G., M.F.C. and T.M. performed the experiments. V.R.M. contributed with specific reagents. F.H.B., A.T., B.K., A.C.M., V.F.P., R.G., V.R.M. and M.A.M.P. analyzed the data. F.H.B., V.F.P. and M.A.M.P. wrote the paper.

Funding

This work was supported by the Canadian Institute of Health Research (MOP 136930, MOP 126000 and MOP 89919; M.A.M.P. and V.F.P.), Canadian Foundation for Innovation (M.A.M.P., V.F.P. and R.G.) and Fundação de Amparo a Pesquisa do Estado de São Paulo, Brazil (FAPESP-2009/14027-2; V.R.M.).

References

Abbas-Terki, T., Briand, P.-A., Donze, O. and Picard, D. (2002). The Hsp90 co-chaperones Cdc37 and Sti1 interact physically and genetically. *Biol. Chem.* **383**, 1335-1342.

Bauman, M. D., Iosif, A.-M., Ashwood, P., Braunschweig, D., Lee, A., Schumann, C. M., Van de Water, J. and Amaral, D. G. (2013). Maternal antibodies from mothers of children with autism alter brain growth and social behavior development in the rhesus monkey. *Transl. Psychiatry* **3**, e278.

Beraldo, F. H., Arantes, C. P., Santos, T. G., Queiroz, N. G. T., Young, K., Rylett, R. J., Markus, R. P., Prado, M. A. M. and Martins, V. R. (2010). Role of alpha7 nicotinic acetylcholine receptor in calcium signaling induced by prion protein interaction with stress-inducible protein 1. *J. Biol. Chem.* **285**, 36542-36550.

Beraldo, F. H., Soares, I. N., Goncalves, D. F., Fan, J., Thomas, A. A., Santos, T. G., Mohammad, A. H., Roffe, M., Calder, M. D., Nikolova, S. et al. (2013). Stress-inducible phosphoprotein 1 has unique cochaperone activity during development and regulates cellular response to ischemia via the prion protein. *FASEB J.* **27**, 3594-3607.

Braunschweig, D., Duncanson, P., Boyce, R., Hansen, R., Ashwood, P., Pessah, I. N., Hertz-Picciotto, I. and Van de Water, J. (2012a). Behavioral correlates of maternal antibody status among children with autism. *J. Autism Dev. Disord.* **42**, 1435-1445.

Braunschweig, D., Golub, M. S., Koenig, C. M., Qi, L., Pessah, I. N., Van de Water, J. and Berman, R. F. (2012b). Maternal autism-associated IgG antibodies delay development and produce anxiety in a mouse gestational transfer model. *J. Neuroimmunol.* **252**, 56-65.

Braunschweig, D., Krakowiak, P., Duncanson, P., Boyce, R., Hansen, R. L., Ashwood, P., Hertz-Picciotto, I., Pessah, I. N. and Van de Water, J. (2013). Autism-specific maternal autoantibodies recognize critical proteins in developing brain. *Transl. Psychiatry* **3**, e277.

Brehme, M., Voisine, C., Rolland, T., Wachi, S., Soper, J. H., Zhu, Y., Orton, K., Villella, A., Garza, D., Vidal, M. et al. (2014). A chaperome subnetwork safeguards proteostasis in aging and neurodegenerative disease. *Cell Rep.* **9**, 1135-1150.

Brimberg, L., Sadiq, A., Gregersen, P. K. and Diamond, B. (2013). Brain-reactive IgG correlates with autoimmunity in mothers of a child with an autism spectrum disorder. *Mol. Psychiatry* **18**, 1171-1177.

Caetano, F. A., Lopes, M. H., Hajj, G. N. M., Machado, C. F., Pinto Arantes, C., Magalhaes, A. C., Vieira, M. d. P. B., Americo, T. A., Massensini, A. R., Priola, S. A. et al. (2008). Endocytosis of prion protein is required for ERK1/2 signaling induced by stress-inducible protein 1. *J. Neurosci.* **28**, 6691-6702.

Camacho, J., Jones, K., Miller, E., Ariza, J., Noctor, S., Van de Water, J. and Martinez-Cerdeno, V. (2014). Embryonic intraventricular exposure to autism-specific maternal autoantibodies produces alterations in autistic-like stereotypical behaviors in offspring mice. *Behav. Brain Res.* **266**, 46-51.

Chen, S., Prapapanich, V., Rimerman, R. A., Honore, B. and Smith, D. F. (1996). Interactions of p60, a mediator of progesterone receptor assembly, with heat shock proteins hsp90 and hsp70. *Mol. Endocrinol.* **10**, 682-693.

Chen, X., Zhao, C., Li, X., Wang, T., Li, Y., Cao, C., Ding, Y., Dong, M., Finci, L., Wang, J.-H. et al. (2015). Terazosin activates Pgk1 and Hsp90 to promote stress resistance. *Nat. Chem. Biol.* **11**, 19-25.

Dalton, P., Deacon, R., Blamire, A., Pike, M., McKinlay, I., Stein, J., Styles, P. and Vincent, A. (2003). Maternal neuronal antibodies associated with autism and a language disorder. *Ann. Neurol.* **53**, 533-537.

Deacon, R. M. J. (2006). Digging and marble burying in mice: simple methods for in vivo identification of biological impacts. *Nat. Protoc.* **1**, 122-124.

Diamond, B., Huerta, P. T., Mina-Osorio, P., Kowal, C. and Volpe, B. T. (2009). Losing your nerves? Maybe it's the antibodies. *Nat. Rev. Immunol.* **9**, 449-456.

Erlich, R. B., Kahn, S. A., Lima, F. R. S., Muras, A. G., Martins, R. A. P., Linden, R., Chiarini, L. B., Martins, V. R. and Moura Neto, V. (2007). STI1 promotes glioma proliferation through MAPK and PI3K pathways. *Glia* **55**, 1690-1698.

Eustace, B. K. and Jay, D. G. (2004). Extracellular roles for the molecular chaperone, hsp90. *Cell Cycle* **3**, 1096-1098.

Flom, G., Weekes, J., Williams, J. J. and Johnson, J. L. (2006). Effect of mutation of the tetratricopeptide repeat and asparatate-proline 2 domains of Sti1 on Hsp90 signaling and interaction in Saccharomyces cerevisiae. *Genetics* **172**, 41-51.

Flom, G., Behal, R. H., Rosen, L., Cole, D. G. and Johnson, J. L. (2007). Definition of the minimal fragments of Sti1 required for dimerization, interaction with Hsp70 and Hsp90 and in vivo functions. *Biochem. J.* **404**, 159-167.

Fox, E., Amaral, D. and Van de Water, J. (2012). Maternal and fetal antibrain antibodies in development and disease. *Dev. Neurobiol.* **72**, 1327-1334.

Gadow, K. D., DeVincent, C. J. and Pomeroy, J. (2006). ADHD symptom subtypes in children with pervasive developmental disorder. *J. Autism Dev. Disord.* **36**, 271-283.

Goldani, A. A. S., Downs, S. R., Widjaja, F., Lawton, B. and Hendren, R. L. (2014). Biomarkers in autism. *Front. Psychiatry* **5**, 100.

Goldstein, S. and Schwebach, A. J. (2004). The comorbidity of Pervasive Developmental Disorder and Attention Deficit Hyperactivity Disorder: results of a retrospective chart review. *J. Autism Dev. Disord.* **34**, 329-339.

Guzman, M. S., De Jaeger, X., Drangova, M., Prado, M. A. M., Gros, R. and Prado, V. F. (2013). Mice with selective elimination of striatal acetylcholine release are lean, show altered energy homeostasis and changed sleep/wake cycle. *J. Neurochem.* **124**, 658-669.

Hajj, G. N. M., Arantes, C. P., Dias, M. V. S., Roffe, M., Costa-Silva, B., Lopes, M. H., Porto-Carreiro, I., Rabachini, T., Lima, F. R., Beraldo, F. H. et al. (2013). The unconventional secretion of stress-inducible protein 1 by a heterogeneous population of extracellular vesicles. *Cell. Mol. Life Sci.* **70**, 3211-3227.

Hashimoto-Torii, K., Torii, M., Fujimoto, M., Nakai, A., El Fatimy, R., Mezger, V., Ju, M. J., Ishii, S., Chao, S.-H., Brennand, K. J. et al. (2014). Roles of heat shock factor 1 in neuronal response to fetal environmental risks and its relevance to brain disorders. *Neuron* **82**, 560-572.

Kolisnyk, B., Guzman, M. S., Raulic, S., Fan, J., Magalhaes, A. C., Feng, G., Gros, R., Prado, V. F. and Prado, M. A. M. (2013). ChAT-ChR2-EYFP mice have enhanced motor endurance but show deficits in attention and several additional cognitive domains. *J. Neurosci.* **33**, 10427-10438.

Lee, D. O. and Ousley, O. Y. (2006). Attention-deficit hyperactivity disorder symptoms in a clinic sample of children and adolescents with pervasive developmental disorders. *J. Child Adolesc. Psychopharmacol.* **16**, 737-746.

Lee, S.-D., Lai, T. W., Lin, S.-Z., Lin, C.-H., Hsu, Y.-H., Li, C.-Y., Wang, H.-J., Lee, W., Su, C.-Y., Yu, Y.-L. et al. (2013). Role of stress-inducible protein-1 in recruitment of bone marrow derived cells into the ischemic brains. *EMBO Mol. Med.* **5**, 1227-1246.

Lima, F. R. S., Arantes, C. P., Muras, A. G., Nomizo, R., Brentani, R. R. and Martins, V. R. (2007). Cellular prion protein expression in astrocytes modulates neuronal survival and differentiation. *J. Neurochem.* **103**, 2164-2176.

Lopes, M. H., Hajj, G. N. M., Muras, A. G., Mancini, G. L., Castro, R. M. P. S., Ribeiro, K. C. B., Brentani, R. R., Linden, R. and Martins, V. R. (2005). Interaction of cellular prion and stress-inducible protein 1 promotes neuritogenesis and neuroprotection by distinct signaling pathways. *J. Neurosci.* **25**, 11330-11339.

Martinez-Cerdeno, V., Camacho, J., Fox, E., Miller, E., Ariza, J., Kienzle, D., Plank, K., Noctor, S. C. and Van de Water, J. (2014). Prenatal exposure to autism-specific maternal autoantibodies alters proliferation of cortical neural

precursor cells, enlarges brain, and increases neuronal size in adult animals. *Cereb. Cortex*. [Epub ahead of print] doi:10.1093/cercor/bhu291.

Martins-Silva, C., De Jaeger, X., Guzman, M. S., Lima, R. D. F., Santos, M. S., Kushmerick, C., Gomez, M. V., Caron, M. G., Prado, M. A. M. and Prado, V. F. (2011). Novel strains of mice deficient for the vesicular acetylcholine transporter: insights on transcriptional regulation and control of locomotor behavior. *PLoS ONE* **6**, e17611.

Martyn, A. C., De Jaeger, X., Magalhaes, A. C., Kesarwani, R., Goncalves, D. F., Raulic, S., Guzman, M. S., Jackson, M. F., Izquierdo, I., Macdonald, J. F. et al. (2012). Elimination of the vesicular acetylcholine transporter in the forebrain causes hyperactivity and deficits in spatial memory and long-term potentiation. *Proc. Natl. Acad. Sci. USA* **109**, 17651-17656.

McFarlane, H. G., Kusek, G. K., Yang, M., Phoenix, J. L., Bolivar, V. J. and Crawley, J. N. (2008). Autism-like behavioral phenotypes in BTBR T+tf/J mice. *Genes Brain Behav.* **7**, 152-163.

Mick, E., McGough, J., Loo, S., Doyle, A. E., Wozniak, J., Wilens, T. E., Smalley, S., McCracken, J., Biederman, J. and Faraone, S. V. (2011). Genome-wide association study of the child behavior checklist dysregulation profile. *J. Am. Acad. Child Adolesc. Psychiatry* **50**, 807-817.e8.

Mulligan, A., Anney, R. J. L., O'Regan, M., Chen, W., Butler, L., Fitzgerald, M., Buitelaar, J., Steinhausen, H.-C., Rothenberger, A., Minderaa, R. et al. (2009). Autism symptoms in Attention-Deficit/Hyperactivity Disorder: a familial trait which correlates with conduct, oppositional defiant, language and motor disorders. *J. Autism Dev. Disord.* **39**, 197-209.

Nicolet, C. M. and Craig, E. A. (1989). Isolation and characterization of STI1, a stress-inducible gene from Saccharomyces cerevisiae. *Mol. Cell. Biol.* **9**, 3638-3646.

Nordahl, C. W., Braunschweig, D., Iosif, A.-M., Lee, A., Rogers, S., Ashwood, P., Amaral, D. G. and Van de Water, J. (2013). Maternal autoantibodies are associated with abnormal brain enlargement in a subgroup of children with autism spectrum disorder. *Brain Behav. Immun.* **30**, 61-65.

Ostapchenko, V. G., Beraldo, F. H., Mohammad, A. H., Xie, Y.-F., Hirata, P. H. F., Magalhaes, A. C., Lamour, G., Li, H., Maciejewski, A., Belrose, J. C. et al. (2013). The prion protein ligand, stress-inducible phosphoprotein 1, regulates amyloid-beta oligomer toxicity. *J. Neurosci.* **33**, 16552-16564.

Picard, D. (2002). Heat-shock protein 90, a chaperone for folding and regulation. *Cell. Mol. Life Sci.* **59**, 1640-1648.

Roffe, M., Beraldo, F. H., Bester, R., Nunziante, M., Bach, C., Mancini, G., Gilch, S., Vorberg, I., Castilho, B. A., Martins, V. R. et al. (2010). Prion protein interaction with stress-inducible protein 1 enhances neuronal protein synthesis via mTOR. *Proc. Natl. Acad. Sci. USA* **107**, 13147-13152.

Romberg, C., Mattson, M. P., Mughal, M. R., Bussey, T. J. and Saksida, L. M. (2011). Impaired attention in the 3xTgAD mouse model of Alzheimer's disease: rescue by donepezil (Aricept). *J. Neurosci.* **31**, 3500-3507.

Scheufler, C., Brinker, A., Bourenkov, G., Pegoraro, S., Moroder, L., Bartunik, H., Hartl, F. U. and Moarefi, I. (2000). Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. *Cell* **101**, 199-210.

Smith, D. F., Sullivan, W. P., Marion, T. N., Zaitsu, K., Madden, B., McCormick, D. J. and Toft, D. O. (1993). Identification of a 60-kilodalton stress-related protein, p60, which interacts with hsp90 and hsp70. *Mol. Cell. Biol.* **13**, 869-876.

Soares, I. N., Caetano, F. A., Pinder, J., Rodrigues, B. R., Beraldo, F. H., Ostapchenko, V. G., Durette, C., Pereira, G. S., Lopes, M. H., Queiroz-Hazarbassanov, N. et al. (2013). Regulation of stress-inducible phosphoprotein 1 nuclear retention by protein inhibitor of activated STAT PIAS1. *Mol. Cell Proteomics* **12**, 3253-3270.

Taipale, M., Jarosz, D. F. and Lindquist, S. (2010). HSP90 at the hub of protein homeostasis: emerging mechanistic insights. *Nat. Rev. Mol. Cell Biol.* **11**, 515-528.

Taipale, M., Tucker, G., Peng, J., Krykbaeva, I., Lin, Z.-Y., Larsen, B., Choi, H., Berger, B., Gingras, A.-C. and Lindquist, S. (2014). A quantitative chaperone interaction network reveals the architecture of cellular protein homeostasis pathways. *Cell* **158**, 434-448.

Vorhees, C. V. and Williams, M. T. (2006). Morris water maze: procedures for assessing spatial and related forms of learning and memory. *Nat. Protoc.* **1**, 848-858.

Wang, T.-H., Chao, A., Tsai, C.-L., Chang, C.-L., Chen, S.-H., Lee, Y.-S., Chen, J.-K., Lin, Y.-J., Chang, P.-Y., Wang, C.-J. et al. (2010). Stress-induced phosphoprotein 1 as a secreted biomarker for human ovarian cancer promotes cancer cell proliferation. *Mol. Cell Proteomics* **9**, 1873-1884.

Zhang, Y., Bolivar, V. J. and Lawrence, D. A. (2012). Developmental exposure to mercury chloride does not impair social behavior of C57BL/6×BTBR F(1) mice. *J. Immunotoxicol.* **9**, 401-410.

Appendix C

Regulation of cognitive processing by hippocampal cholinergic tone

This is a copyedited, author-produced PDF of an article accepted for publication in *Cerebral Cortex* following peer review.

Al-Onaizi, MA, Parfitt, GM, Kolisnyk, B, Law, CSH, Guzman, MS, Barros, DM, Leung, SL, Prado, MAM, Prado, VF “Regulation of cognitive processing by hippocampal cholinergic tone”. Cerebral Cortex, piibhv349 (2016) [Epub ahead of print]

Contributions to publication: BK performed working memory experiments with the VAChTNkx2.1-Cre-flox/flox animals as well as the PAL experiments in the AAV8-Cre injected animals.

OXFORD

Cerebral Cortex, 2016, 1–14

doi: 10.1093/cercor/bhv349
Original Article

ORIGINAL ARTICLE

Regulation of Cognitive Processing by Hippocampal Cholinergic Tone

Mohammed A. Al-Onaizi[1,2,†], Gustavo M. Parfitt[1,5,†], Benjamin Kolisnyk[1,3], Clayton S. H. Law[4], Monica S. Guzman[1,4], Daniela Martí Barros[5], L. Stan Leung[4], Marco A. M. Prado[1,2,3,4] and Vania F. Prado[1,2,3,4]

[redacted]

Address correspondence to Marco A. M. Prado. Email: [redacted]

[†]These authors contributed equally.

Abstract

Cholinergic dysfunction has been associated with cognitive abnormalities in a variety of neurodegenerative and neuropsychiatric diseases. Here we tested how information processing is regulated by cholinergic tone in genetically modified mice targeting the vesicular acetylcholine transporter (VAChT), a protein required for acetylcholine release. We measured long-term potentiation of Schaffer collateral-CA1 synapses in vivo and assessed information processing by using a mouse touchscreen version of paired associates learning task (PAL). Acquisition of information in the mouse PAL task correlated to levels of hippocampal VAChT, suggesting a critical role for cholinergic tone. Accordingly, synaptic plasticity in the hippocampus in vivo was disturbed, but not completely abolished, by decreased hippocampal cholinergic signaling. Disrupted forebrain cholinergic signaling also affected working memory, a result reproduced by selectively decreasing VAChT in the hippocampus. In contrast, spatial memory was relatively preserved, whereas reversal spatial memory was sensitive to decreased hippocampal cholinergic signaling. This work provides a refined roadmap of how synaptically secreted acetylcholine influences distinct behaviors and suggests that distinct forms of cognitive processing may be regulated in different ways by cholinergic activity.

Key words: Alzheimer's disease, long-term potentiation, Morris water maze, paired associates learning, vesicular acetylcholine transporter, working memory

Introduction

Basal forebrain cholinergic neurons provide input to the entire cortex and hippocampus. In particular, the hippocampus receives most of its cholinergic innervation from neurons in the medial septal nucleus (MS) and vertical limb of the diagonal band of Broca (vdB), whereas the cerebral cortex and the amygdala receive cholinergic inputs from neurons located in the nucleus basalis of Meynert (NBM) (Mesulam et al. 1992; Kitt et al. 1994). Abnormalities in forebrain cholinergic nuclei have been implicated in several cognitive disorders (Bartus 2000; Mesulam 2004), including Alzheimer's disease (AD ; Grothe, Schuster, et al. 2014; Teipel et al. 2014). Moreover, cumulative use of drugs with anticholinergic activity is associated with increased risk for dementia and AD (Gray et al. 2015). However, the relationship between cholinergic dysfunction and maintenance of cognitive abilities in these diseases is not fully understood, due to concomitant pathologies that may contribute to cognitive abnormalities (Mesulam 2013).

Cholinergic signaling is involved in the regulation of hippocampal synaptic transmission and plasticity (Ji et al. 2001;

Leung et al. 2003; Seeger et al. 2004; Ge and Dani 2005; Gu and Yakel 2011). Septal cholinergic activation, either by electrical stimulation or by optogenetics, allows the expression of distinct time-dependent forms of hippocampal plasticity (Gu and Yakel 2011). Pharmacological (Decker and Majchrzak 1992; Anagnostaras et al. 1999; Gale et al. 2001; Wallenstein and Vago 2001; Chudasama et al. 2004; Pichat et al. 2007; Timmermann et al. 2007; Ragozzino et al. 2012) and genetic studies (Anagnostaras et al. 2003; Seeger et al. 2004; Poulin et al. 2010) have shown that modulation of cholinergic receptors influence learning and memory processes. Indeed, both nicotinic receptors (nAChRs) and muscarinic receptors (mAChRs) have been linked with various forms of plasticity (Vidal and Changeux 1993; Gray et al. 1996; Ji and Dani 2000; Seeger et al. 2004; Gautam et al. 2006; Giessel and Sabatini 2010; Zheng et al. 2012). For example, M1 knockout mice exhibit selective cognitive impairments in tasks requiring interactions between the hippocampus and cortex (Anagnostaras et al. 2003), while M2 knockout mice display impairments in working memory, cognitive flexibility, and hippocampal plasticity (Seeger et al. 2004). Moreover, recent evidence shows that activation of M1 mAChRs induces long-term potentiation (LTP), suggesting that M1 mAChRs could play a role in regulating hippocampal plasticity (Dennis et al. 2015). Furthermore, the nAChR β2-subunit knockout mice are impaired in learning the MWM, suggesting that the β2-subunit may mediate effects of ACh on learning and memory (Zoli et al. 1999). However, long-term changes in cholinergic activity, as observed in a number of neurodegenerative diseases, are more complex to model using specific receptor knockouts, given the plethora of subtypes of muscarinic and nicotinic receptors.

One widespread alternative to mimic cholinergic dysfunction is the selective elimination of these neurons using toxins in rodents (Baxter and Bucci 2013; Prado et al. 2013). It is somewhat controversial whether selective 192 IgG-saporin lesion of septohippocampal cholinergic neurons can lead to significant impairments in hippocampal-dependent learning and memory tasks in rodents, with some authors finding little effect (Berger-Sweeney et al. 1994; Baxter and Gallagher 1996; Pizzo et al. 2002; Frick et al. 2004; Parent and Baxter 2004), whereas others find a miriad of deficits (Walsh et al. 1996; Janis et al. 1998; Gil-Bea et al. 2011). In addition, cholinergic neurons have been shown to contain more than one class of neurotransmitter transporters and secrete 2 neurotransmitters (Gras et al. 2008; El Mestikawy et al. 2011; Guzman et al. 2011; Prado et al. 2013; Nelson et al. 2014; Saunders et al. 2015). Therefore, it is difficult to interpret results with toxin lesions for specific contributions of neurotransmitters in neurons that release 2 chemical messengers. Indeed, recent work has shown that some basal forebrain cholinergic neurons can also secrete GABA which acts as a neurotransmitter in the cortex (Saunders et al. 2015).

Genetic targeting of either the vesicular acetylcholine transporter (VAChT; Guzman et al. 2011; Martyn et al. 2012) or choline acetyltransferase (ChAT; Patel et al. 2012) using the Cre/lox system has provided a way for investigating specific contributions of ACh when there is co-transmission (Prado et al. 2013). Decreased VAChT levels severely compromise packaging of acetylcholine (ACh) into synaptic vesicles and thus reduce ACh release by nerve terminals (Prado et al. 2006; de Castro, De Jaeger, et al. 2009). Conversely, overexpression of VAChT enhances ACh secretion proportionally (Song et al. 1997; Kolisnyk, Guzman, et al. 2013) .

The recent development of automated touchscreen behavioral testing for rodents has greatly improved mouse behavioral assessment. Touchscreen tasks were designed using almost identical paradigms and methodologies used in humans, facilitating translational studies between species (Morton et al. 2006; Talpos et al. 2009, 2010; Romberg et al. 2011; Mar et al. 2013). The paired associates learning (PAL) test has been shown to efficiently detect cognitive alterations that are consistently observed in AD (Swainson et al. 2001; Blackwell et al. 2004; de Rover et al. 2011) and schizophrenia (Wood et al. 2002; Barnett et al. 2005). In dementia, PAL has been shown to differentiate between mild cognitive impairment and AD (Blackwell et al. 2004). Here we investigated cognitive performance in mice with deletion of VAChT, a protein required for synaptic release of ACh, in either forebrain cholinergic neurons or selectively in septohippocampal cholinergic neurons. Our experiments reveal that dysfunction in hippocampal cholinergic activity influences synaptic plasticity in vivo and disturbs performance in PAL and working memory, whereas spatial navigation seems relatively preserved.

Material and Methods

Animals

Generation of VAChT$^{flox/flox}$ mice was previously described (Martins-Silva et al. 2011). VAChT$^{flox/flox}$ mice (mixed C57BL/6J × 129/SvEv background, backcrossed to C57BL/6J for 5 generations) were crossed to VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice so that offspring from this mating provided control and test littermates. VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice were generated by crossing VAChT$^{flox/flox}$ with the Nkx2.1-Cre mouse line (C57BL/6J-Tg(Nkx2-1-cre)2Sand/J), purchased from The Jackson Laboratory (JAX stock no. 008661). This line has been previously used to eliminate ChAT from forebrain neurons (Patel et al. 2012). Unless otherwise stated, all control mice used for behavioral studies were VAChT$^{flox/flox}$ littermates. The reporter mouse line Nkx2.1$^{(td\text{-}Tomato)}$ was generated by crossing B6.Cg-Gt(ROSA)26Sor$^{tm9(CAG\text{-}tdTomato)Hze}$/J mice, purchased from The Jackson Laboratory (JAX stock no. 007909) with the Nkx2.1-Cre mouse line (JAX stock no. 008661).

Animals were housed in groups of 3 per cage without environmental enrichment in a temperature controlled room (12:12 light to dark cycles), and food and water were provided ad libitum for most experiments. Animals that underwent touchscreen testing were housed in pairs; food restricted to no more than 85% of their original weight, and they were maintained at the target weight for the duration of behavioral testing. Male mice 3 months old were used for behavioral studies. We followed the ARRIVE guidelines (Kilkenny et al. 2010); hence, mice were randomized for behavioral tests and the experimenter was blind to the genotype. All procedures were performed in accordance with the Canadian Council of Animal Care guidelines at the University of Western Ontario with an approved animal protocol (2008–127).

Immunoflourescence Microscopy

Mice were anesthetized with ketamine (100 mg/kg) and xylazine (25 mg/kg) in 0.9% sodium chloride, and then sacrificed by transcardial perfusion: phosphate-buffered saline (PBS, pH = 7.4) for 3 min and 4% paraformaldehyde for 5 min. Brains were harvested and placed in 4% paraformaldehyde in 1× PBS at 4 °C for 4 h; they were kept at 4 °C until sliced using a vibratome. Brain sections (40 µm) were prepared and free-floating sections in 1× PBS (1 per well in a 24-well plate) were permeabilized with 0.4% Triton X-100 in 1× PBS for 1 h. Non-specific epitopes were blocked using a solution of 1× PBS/0.4% Triton X-100 containing 0.1% glycine (wt/vol), 0.1% lysine (wt/vol), 1% BSA (wt/vol), and 1% normal donkey serum (wt/vol). The primary antibodies used were anti-VAChT (catalog no. 139103; Synaptic Systems), anti-ChAT

(1:200) (catalog no. AB144P, Merck Millipore), and anti-Choline Transporter (CHT1; 1:200), which was kindly donated by Dr R. Jane Rylett, University of Western Ontario, London, Ontario. The primary antibody was incubated in blocking buffer overnight at 4 °C. Sections were then washed 5 times in 1× PBS/0.4% Triton X-100 (10 min each). Hoechst 3342 (Life Technologies, Bibco, Carlsbad, CA, USA) (2–5 µg/mL) and secondary antibodies (1:500; anti-488, catalog no. A-11034, ThermoFisher; 1:500 anti-633, catalog no. A-21082, ThermoFisher) were diluted in blocking buffer and slices were incubated for 1 h at RT. Sections were visualized by Zeiss LSM 510Meta (Carl Zeiss, Oberkochen, Germany) confocal system (63 × objective, 488-nm Ar laser and 633-nm HeNe laser were used for excitation of fluorophores).

Western Blotting

Immunoblotting was performed as previously described (Martins-Silva et al. 2011; Kolisnyk, Al-Onaizi, et al. 2013; Kolisnyk, Guzman, et al. 2013). Antibodies used were anti-VAChT (catalog no. 139103; Synaptic Systems) and anti-Synaptophysin (catalog no. S5768; Sigma-Aldrich).

Training on the PAL Task

Prior to training, both groups of mice (3 months old) were food restricted until they reached approximately 85% of their original weight. Training of the animals to the PAL task was previously described (Talpos et al. 2009). Briefly, the training phase for the mice in the touchscreen chambers involved a habituation session, where they were placed in the chambers with the lights off for 20 min to habituate to the environment for 2 days. Next, mice were put in the chamber with the same parameters as in the habituation phase, but this time a 150 µL reward (strawberry milkshake; Saputo Dairy Products, Canada) was introduced in the reward receptacle. Every time the mouse attended to the reward in the reward receptacle, a tone was played. This 40 min training session was done for the next 2 days until mice completed 36 trials in 60 min (Habituation; Phase 1)

The mice were then trained to associate the reward with a 30 s presentation of training stimuli, which varied in brightness, shape, and pattern, on one of the 3 screens (Initial touch training; Phase 2). Mice were required to touch any of the screens whenever the stimulus was presented to receive the reward, which was paired with a tone. A new trial was automatically initiated once the mice collected the reward. This was done until the mice completed 36 trials in 60 min for 1 day. Next, mice are required to touch the stimulus that is displayed randomly in one of the 3 windows to receive the reward (must touch stimuli training; Phase 3). Mice are only moved to next training after completing 36 trials in 60 min for 1 day. In the next training phase, food is delivered and tray light is turned on. The mouse must nose poke and exit the reward tray before a stimulus is displayed randomly on the screen (Must initiate; Phase 4). This was done until mice completed 36 trials in 60 min for 1 day. Next, animals go to the last phase of the training program required for the PAL task. This training phase is referred to as "punish incorrect" (Phase 5). This phase is similar to the previous one, but if the mouse touched the incorrect screen, that is, one of the blank screens, it was presented with a 5-s time-out. This time-out was accompanied by the presentation of a bright light in the chamber. Criterion to successfully proceed from this training phase was 23 correct responses out of 30 trials in 60 min for 2 consecutive days. Next, both experimental groups were subjected to acquisition training, where 2 stimuli are displayed at the same time during a trial. One will be in the correct location (S+) and the other will be in the incorrect location (S–). Mice were required to touch the correct stimulus (S+) presented on one of the 3 screens to complete a trial and receive the reward. In this acquisition phase, mice were on an unpunished version in which touching the S– was ignored. A completion of a trial was only considered when the mouse touches the S+. Criterion for this training phase is completion of 36 trials in 1 session (1 day). All mice from both experimental groups were able to reach criterion in acquisition training.

PAL Task

After successfully completing the training phase, the mice were placed on a PAL task (dPAL), which involves a different stimulus being presented in each trial. A trial starts in dPAL when the mouse initiates it by touching the food receptacle, which triggers the display of both S+ and S– on the screen. S+ refers to when the stimulus is in the correct location, and S– refers to when the stimulus is in the incorrect location. There were 6 possible trial types and 3 different stimuli were presented (flower, plane, and spider). Within trials, an S+ is the flower presented in the left window, the plane in the middle window, or the spider in the right window. Thus, mice are required to learn to associate a stimulus to its correct location. A response by touching the S– resulted in a 10 s time-out and the chamber light was activated for 10 s, acting as an indication for an incorrect response for the mouse. After 10 s, the next initiation by the mouse was considered a correction error trial, where the same S+ and S– were presented as for the unsuccessful previous trial. The number of correction trials was not counted toward the total number of trials performed per session. An S+ response, however, led to a tone, as well as the reward being dispensed in the receptacle.

Electrophysiology

Animals were anesthetized with urethane (1 g/kg i.p.) and placed in a stereotaxic apparatus. Atropine methyl nitrate was administered (5 mg/kg i.p.) to reduce airway secretions during stereotaxic surgery. Animal body temperature was monitored between 36.5 °C and 37 °C using a feedback controlled rectal thermometer and heating pad. Stimulating electrodes were placed into stratum radiatum at P 1.8, L 2.3 or P 2.5, L2.4 (Franklin and Paxinos 2008) to stimulate Schaeffer collaterals projecting from CA3 to CA1 (Hutchison et al. 2009). A silicon probe, with 16 electrodes separated by 50 µm on a vertical shank, was placed in area CA1 at P 2.2, L 1.8. Laminar profiles of the average (4 sweeps) field excitatory postsynaptic potentials evoked by single pulse stimulation of the Schaffer collaterals at 1.5-2 × threshold stimulus intensity. Current-source density analysis using 100 µm step size was used to determine current sources and sinks. The maximal slope (of 1 ms duration) during the rising phase of the excitatory sink, at its maximum in CA1 stratum radiatum, was used for LTP assessment. After a stable baseline of the excitatory sink slope was established for 30 min (coefficient of variation [SEM/mean] of the sink slopes <0.05), a high-frequency tetanus (100 Hz for 1 s) was delivered at 2–3 times the threshold intensity, and the response was measured for 120 min after the tetanus. For each mouse, the slope of the excitatory sink was normalized by the average value of the baseline, and LTP across mice was averaged and reported as a multiple of the baseline slope.

Rotarod and Neuromuscular Tests

The rotarod task was conducted as previously described (Prado et al. 2006; de Castro, Pereira, et al. 2009). Forelimb and hind

limb grip strength was assessed using a previously described protocol (Prado et al. 2006). The hang-wire experiments were performed as described (Sango et al. 1996).

Morris Water Maze

The spatial version of the MWM was performed as previously described (Vorhees and Williams 2006; Martyn et al. 2012; Kolisnyk, Guzman, et al. 2013). Testing was performed in a 1.5-m diameter pool with 25 °C water. A hidden platform was submerged in a constant location 1 cm below the surface of the water in one of the 4 arbitrarily defined quadrants, and spatial cues were distributed around the pool. Briefly, mice were given four 90-s trials for the duration of 4 days to find the hidden platform, with an ITI of 15 min. The animals were introduced to the pool from different locations within the pool for each trial. Mice that did not find the platform within the 90 s were gently guided to the platform. On the fifth day, spatial memory recall was tested by a 60-s probe trial, where the hidden platform is removed and the amount of time the animal spends in the target quadrant is calculated. To test reversal learning, the hidden platform was relocated to the opposite quadrant, where the animals were given four 90-s trials for 4 days. On the fifth day, the animals were given a 60-s probe trial. Data were analyzed using ANY-Maze video tracking software (Stoelting Co.).

Two-Trial Morris Water Maze

A task used to assess working memory was the 2-trial variation of the MWM. The task was carried out using previously described protocols (Vorhees and Williams 2006; Kolisnyk, Guzman, et al. 2013). The mice were trained on the task over the course of 5 days. During the training period, the mouse was first given a 90-s trial with a 15 s inter-trial interval. Next, the mouse was given a second trial with the same platform location and starting point; this was repeated 3 additional times. After completing the training phase, the mouse was first given a 90 s trial with a 15 s inter-trial-interval. The mouse was then given a second trial with identical platform location and starting point. This was repeated with 4 unique starting location/platform location combinations a day. Mean latency and distance savings ratios were then calculated as previously described (Kolisnyk, Guzman, et al. 2013). Sessions were recorded for both tests and were analyzed using the ANY-Maze video tracking software (Stoelting Co.)

Spontaneous Alterations Y-maze

To assess working memory in the mice, we used the spontaneous alternations Y-maze as previously described (Kolisnyk, Guzman, et al. 2013). Briefly, mice were placed in a symmetrical plastic Y-maze apparatus, and both the number and order of arm entries were recorded. A spontaneous alternation was defined as when the mouse visited all 3 of the arms in a row, without having revisited a previous arm of the maze. Sessions were recorded and analyzed using the ANY-Maze Software.

Stereotaxic Injections of Adeno-Associated Virus

To obtain selective deletion of VAChT in the medial septum, mice were anesthetized with ketamine (100 mg/kg) and xylazine (25 mg/kg) in 0.9% sodium chloride, and 1 µL (titer of ~10^{13} GC/mL) of adeno-associated virus (AAV)8-GFP-Cre- or control virus (AAV8-GFP, Vector BioLabs, Eagleville, PA, USA) was injected into the medial septum/vertical limb of the diagonal band (0.98 AP, 0.1 LL and 4.1 DV) of VAChT$^{flox/flox}$ mice. The injecting micropipette was inserted and left for 2 min to stabilize. After stabilization, a 0.2 µL/min infusion was performed using a micropump followed by a 30 min rest period to allow local diffusion of the virus and avoid virus efflux. The micropipette was then slowly removed and the scalp sutured. A recovery period of 4 weeks was given before behavioral testing to allow transgene expression.

Statistical Analysis

All data are expressed as mean ± SEM. Sigmastat 3.5 software was used for statistical analysis. Comparison between 2 experimental groups was done with Student's *t*-test. When several experimental groups or treatments were analyzed, 2-way analysis of variance (ANOVA) or 2-way ANOVA with repeated measures (RM) were used as required. When appropriate, a Bonferonni *post hoc* analysis test was used.

Results

Deletion of VAChT in Forebrain Projection Neurons

Nkx2.1-driven Cre is expressed in forebrain cholinergic neurons as assessed using a reporter mouse line (see Supplementary Fig. 1A and Table 1). Immunoblot analysis shows that VAChT levels in the prefrontal cortex ($t_{(4)} = 6.162$, $P = 0.0035$), hippocampus ($t_{(4)} = 4.461$, $P = 0.0097$), and striatum ($t_{(4)} = 8.625$, $P = 0.0010$) were severely diminished in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice (see Supplementary Fig. 1B–D). In contrast, VAChT levels remained unchanged in the brainstem of VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ compared with controls ($t_{(4)} = 1.040$, $P = 0.3571$, see Supplementary Fig. 1E). Moreover, immunofluorescence imaging indicated decreased VAChT immunoreactivity in the hippocampus of VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice compared with controls (Fig. 1A,B). Importantly, these mice presented no neuromuscular deficits (see Supplementary Fig. 2A–C). We have previously shown that reduced VAChT levels proportionally decrease the release of ACh in vivo and in vitro (Prado et al. 2006; Guzman et al. 2011; Kolisnyk, Al-Onaizi, et al. 2013; Kolisnyk, Guzman, et al. 2013).

Forebrain VAChT is Required for Performance in the PAL Task

We tested VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice on the PAL task, which requires sophisticated processing of information for proper association of images with specific locations. VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice and their matched controls were assessed on the dPAL task using an automated touchscreen system (Fig. 1C and see Supplementary Videos 3 and 4). Prior to being subjected to the PAL task, both experimental groups are trained on a different training sessions (initial touch, must touch stimuli, must initiate, and punish incorrect) to learn how to operate the touchscreen, which includes learning to touch the screen when a stimulus is presented and initiating the task by inserting the head into the reward chamber. In the "punish incorrect" training, when only one stimulus is presented randomly in one of the 3 screens, mice are taught to touch the screen that shows the stimulus. Mice from both experimental groups were able to reach criterion in this phase of the training and no differences were observed between the 2 genotypes ($t_{(12)} = 0.0749$) (Fig. 1D), indicating that VAChT-deficient mice are able to learn that they need to touch the screen when an image is shown. Additionally it argues that VAChT-deficient mice do not present any major visual impairment. During the course of the 9 weeks that mice were tested on the dPAL task, we observed that control mice significantly improved their accuracy performance, while VAChT deletion

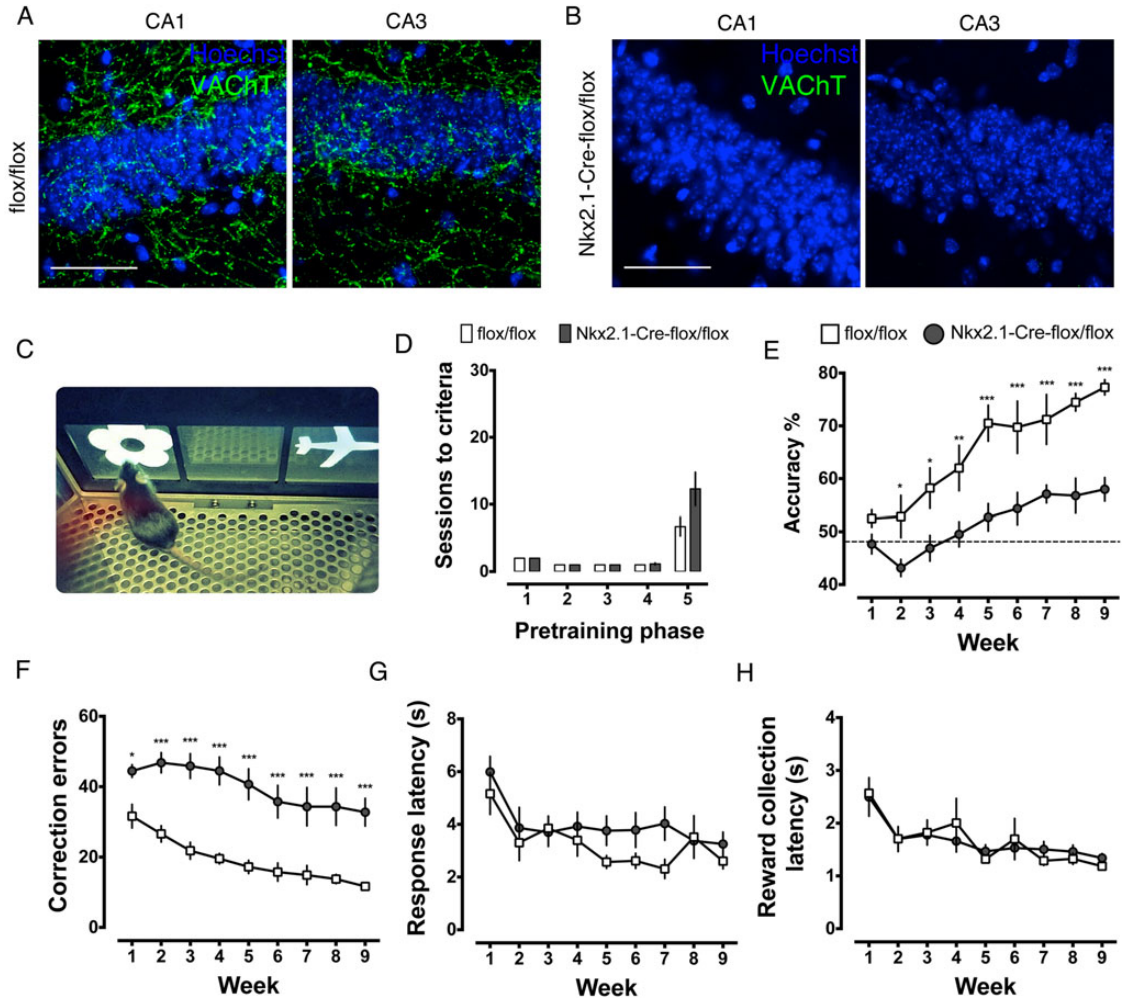


Figure 1. VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ mice display impairments in the acquisition of dPAL. (*A*) Representative 3-dimensional reconstructed *Z* stack immunofluorescence images of VAChT (green) and Hoechst (blue) in the CA1 and CA3 regions of the hippocampus in VAChT$^{flox/flox}$ ($n = 3$) and (*B*) VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice ($n = 3$) (Scale bar = 100 μm). (*C*) Image depicting a mouse performing the task, where the flower is shown as S+ and the airplane as S−. (*D*) Number of sessions required by both experimental groups to reach criterion during the operant conditioning, pretraining, and training phases. (*E–H*) Data for the acquisition of the dPAL task for VAChT$^{flox/flox}$ ($n = 7$ clear squares) and VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ ($n = 7$ dark circles) mice. Each week represents 5 testing sessions of 36 trials. (*E*) Mean accuracy; (*F*) Mean correction errors; (*G*) Response latency; (*H*) Reward collection latency (Data are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$).

mutants did not (2-way RM ANOVA shows significant effect of weeks $F_{8,48} = 21.11$, $P < 0.0001$, an effect of genotype $F_{1,6} = 56.94$, $P = 0.0003$, and an interaction effect $F_{8,48} = 2.871$, $P = 0.0074$, Fig. 1*E*). VAChT$^{flox/flox}$ mice (controls) were able to improve performance reaching 77 ± 1% accuracy by Week 9 (Fig. 1*E*). In contrast, peak accuracy performance of VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice in the dPAL task during the same period was 58 ± 2% (Fig. 1*E*). Although VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice were able to perform the 36 trials required in each 1-h session, they failed to associate the stimulus to its correct location. Their poorer performance was also reflected in the number of correction errors performed (Fig. 1*F*). VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice failed to decrease the number of correction errors made over the course of 9 weeks, while control mice improved the number of correction errors performed during the course of the study (2-way RM ANOVA shows significant effect of weeks $F_{8,48} = 12.05$, $P < 0.0001$, an effect of genotype $F_{1,6} = 39.41$, $P = 0.0008$, and an interaction effect $F_{8,48} = 1.224$, $P = 0.0306$, Fig. 1*F*). Correct response latency was not different between the 2 groups over the course of 9 weeks (2-way RM ANOVA shows significant effect of weeks $F_{8,48} = 7.508$, $P < 0.0001$, no effect of genotype $F_{1,6} = 2.437$, $P = 0.1695$, and no interaction $F_{8,48} = 1.195$, $P = 0.3220$, Fig. 1*G*). Furthermore, VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice were no different from controls when the latency to collect the reward was measured, which indicated that motivation was not a factor in their poorer performance (2-way RM ANOVA shows a significant effect of weeks $F_{8,48} = 7.596$, $P < 0.0001$, no effect of genotype $F_{1,6} = 0.0001380$, $P = 0.7681$, and no interaction $F_{8,48} = 0.6061$, $P = 0.7681$ Fig. 1*H*). In summary, VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice were able to learn that they had to touch the screen when the images were shown; however, they failed in making associations, that is, they were unable to assign each image to a specific position.

Hippocampal LTP is Disrupted in Forebrain-Specific VAChT Knockout Mice In Vivo

Formation of associations might depend on lasting increases in synaptic strength. To determine whether VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice have intact synaptic plasticity, we examined LTP of the synapse of the Schaffer collaterals on hippocampal CA1 neurons in anaesthetized mice in vivo. VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice showed decreased LTP which lasted about 90 min post-tetanus delivery while LTP in VAChT$^{flox/flox}$ mice was maintained for 120 min (Fig. 2*A*,*B*). This indicated that the lack of cholinergic signaling disturbs synaptic plasticity in hippocampal CA1 area in vivo.

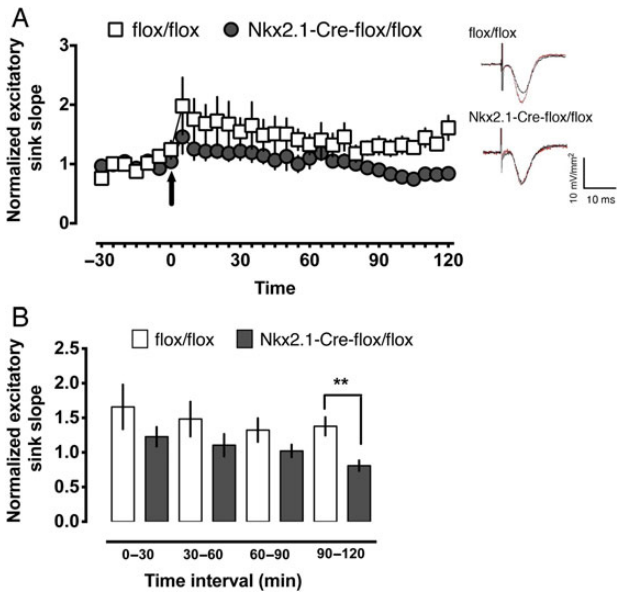


Figure 2. Hippocampal LTP is disrupted in forebrain-specific VAChT knockout mice in vivo. (*A*) Normalized slopes of the excitatory sink recorded at CA1 stratum radiatum (apical dendrites) of VAChT$^{flox/flox}$ (clear squares, $n = 5$) and VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ (dark circles, $n = 6$) mice. Baseline was monitored for 30 min prior to tetanus delivery ($t = 0$), and posttetanic response was monitored for 120 min. A 1-s 100 Hz train, delivered at 2–3 times the threshold intensity (arrow), induced higher and more prolonged potentiation in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice than VAChT$^{flox/flox}$ controls. Insets show representative current sink time response taken at 80 min (red traces), overlaid on the pretetanus baseline response (black traces), from each genotype. (*B*) Normalized excitatory sink slope averaged across 30-min time intervals (mean ± SEM) in VAChT$^{flox/flox}$ and VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice, with significant difference between mouse groups at 90–120 min ($t_{(9)} = 3.911$, $P = 0.0036$).

To specifically evaluate the contribution of hippocampal cholinergic tone to PAL performance, we stereotaxically injected AAV8-GFP-Cre or AAV8-GFP virus to the medial septum and vertical limb of the diagonal band (MS/VDB) of VAChT$^{flox/flox}$ mice (AAV8-GFP-Cre $n = 13$; AAV8-GFP $n = 7$). Mice were trained on the dPAL task 1 month after viral injection. Following completion of the task, mice were sacrificed to evaluate VAChT protein levels. Given the length of the experiment (≈4 months), and the observation that viral injection was only partially effective to reduce hippocampal VAChT levels (see Supplementary Fig. 6*B*), we did not exclude any mouse from the analysis, even if viral mediated recombination was not effective to eliminate the transporter. Instead, we correlated VAChT levels in the hippocampus from both AAV8-GFP-Cre and AAV8-GFP to their performance on the PAL task.

Performance on the final week of the experiment was positively correlated to VAChT protein levels in terms of response accuracy (Pearson's $r = 0.5208$, CI = 0.1015–0.7829, $P = 0.0186$, Fig. 3*A*) and negatively correlated to number of correction errors (Pearson's $r = -0.6518$, CI = −0.8494 to −0.2940, $P = 0.0018$, Fig. 3*B*). We also evaluated the relationship between hippocampal VAChT protein levels to learning the PAL task. We calculated the rate of learning as the slope of the learning curve of both response accuracy and correction errors across all the weeks of the task. VAChT protein level was positively correlated to the rate of learning of response accuracy (Pearson's $r = 0.5053$, CI = 0.08072–0.7747, $P = 0.0231$, Fig. 3*C*) and negatively correlated to the correction error rate of learning (Pearson's $r = -0.1799$, CI = −0.7982 to −0.1418, $P = 0.0120$, Fig. 3*D*). Importantly, VAChT protein levels did not correlate to mean response latency across the task (Pearson's $r = 0.1349$, CI = −0.3273 to 0.5450, $P = 0.5708$, Fig. 3*E*) or mean reward collection latency across the task (Pearson's $r = -0.1799$, CI = −0.5676 to 0.2731, $P = 0.4352$, Fig. 3*F*), suggesting that response patterns and motivation are unaltered by reduced VAChT levels. Taken together these results show that the less VAChT protein in the hippocampus the worse is the mouse performance in the dPAL task, indicating that dPAL learning is modulated by septohippocampal cholinergic signaling.

VAChT and Spatial Navigation

Given the strong deficit of association of the image with its correct location in the PAL task, it seemed of importance also to evaluate spatial memory in these mice. Spatial memory is widely used to assess information acquisition and storage in the hippocampus, but cholinergic dysfunction has only mild effects in the MWM in mice (Moreau et al. 2008; Martyn et al. 2012). Our data showed that spatial learning on the MWM was relatively normal in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice compared with controls (see Supplementary Fig. 5*A–C*). On the probe trial of the MWM, both groups of mice spent significantly more time in the target quadrant compared with the opposite quadrant (2-way ANOVA shows a significant effect of quadrant, $F_{3,80} = 39.58$, $P < 0.0001$, and an interaction effect $F_{3,80} = 2.914$, $P = 0.0394$, see Supplementary Fig. 5*D*), *post hoc* analysis revealed that both groups spent significantly more time in the target quadrant. However, VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice had significantly fewer platform crosses compared with littermate controls ($t_{(20)} = 2.795$, $P = 0.0112$, see Supplementary Fig. 5*E*).

To specifically evaluate the contribution of hippocampal cholinergic tone to learning and memory performance in the spatial version of the MWM, we stereotaxically injected AAV8-GFP-Cre ($n = 25$) virus to the MS/VDB in another cohort of VAChT$^{flox/flox}$ mice (see Supplementary Fig. 6*A,B*). VAChT$^{flox/flox}$ mice injected with AAV8-GFP ($n = 14$) were used as controls. AAV8-GFP-Cre-injected mice that showed more than 50% of hippocampal VAChT protein levels ($n = 11$) compared with controls were excluded from the analysis (see Supplementary Fig. 6*E*). In AAV8-GFP-Cre-injected mice with reduced hippocampal VAChT levels, VAChT protein in the prefrontal cortex was not changed (97% of AAV8-GFP VAChT levels, $t_{(4)} = 0.453$, $P = 0.665$, see Supplementary Figure 6*C,D*). AAV8-GFP-Cre-mediated deletion of VAChT from the medial septum did not significantly alter acquisition of the spatial version of the MWM (Latency, 2-way RM ANOVA shows an effect of days $F_{3,39} = 22.84$, $P < 0.0001$, no effect of Cre virus injection $F_{1,13} = 0.2228$, $P = 0.6447$, and no interaction, $F_{3,39} = 1.302$, $P = 0.2876$, Fig. 4*A*). Similar results were obtained for distance travelled (2-way RM ANOVA shows an effect of days, $F_{3,39} = 23.5$, $P < 0.0001$, no effect of Cre expression $F_{1,13} = 0.3125$, $P = 0.5856$, and no interaction, $F_{3,39} = 1.329$, $P = 0.2787$, Fig. 4*B*). In the probe trial, mice injected with the AAV8-GFP-Cre virus did not differ from controls in terms of preference for the target quadrant (2-way ANOVA shows a significant effect of quadrant, $F_{3,104} = 37.81$, $P < 0.0001$, no effect of Cre expression, $F_{1,104} = 0.6452$, $P = 0.4237$, and no interaction $F_{3,104} = 0.3988$, $P = 0.7541$, Fig. 4*D*) or platform crosses ($t_{(26)} = 0.9547$, $P = 0.3603$, Figure 4*E*). Taken together, these results suggest that decreased levels of hippocampal cholinergic activity do not seem to affect MWM performance.

VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice were also tested on the reversal learning protocol of the MWM. During the course of 4 days, control mice significantly improved in their latency to find the hidden platform in contrast to VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice (2-way RM ANOVA shows a significant effect of days $F_{3,30} = 8.632$, $P = 0.0003$, main effect of genotype $F_{1,10} = 11.17$, $P = 0.0075$, and

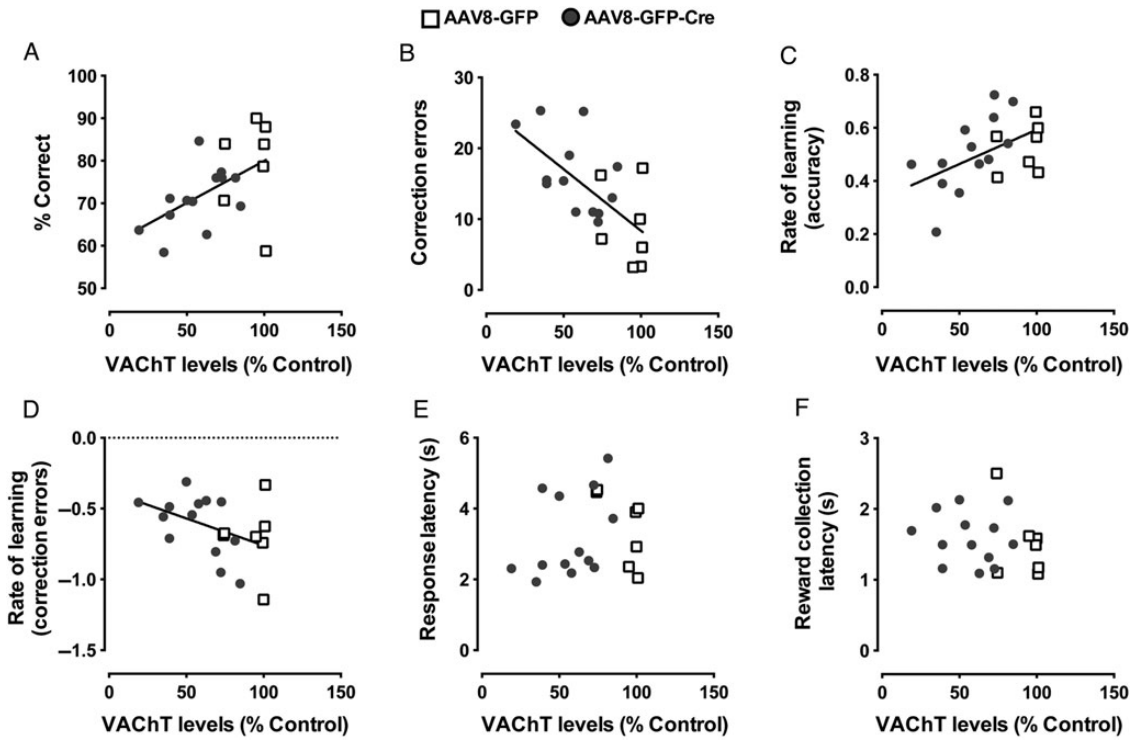


Figure 3. Medial septum AAV8-GFP-Cre-injected mice show deficits in dPAL. (*A*,*B*) Linear regression and correlation between response accuracy ($r = 0.5208$, $P = 0.0186$) and correction errors ($r = -0.5154$, $P = 0.0168$) on Week 9 and hippocampal VAChT protein expression levels for AAV8-GFP (clear squares, $n = 7$) and AAV8-GFP-Cre (dark circles, $n = 13$) injected mice. (*C*,*D*) Linear regression and correlation between response accuracy ($r = 0.4460$, $P = 0.0487$) and correction errors ($r = -0.1799$, $P = 0.0120$) across all the weeks of the PAL task and hippocampal VAChT protein expression levels. (*E*,*F*) The relationship between response latency ($r = 0.1349$, $P = 0.5708$) and reward collection latency ($r = -0.1799$, $P = 0.4352$) across all the weeks of the PAL task and VAChT expression levels.

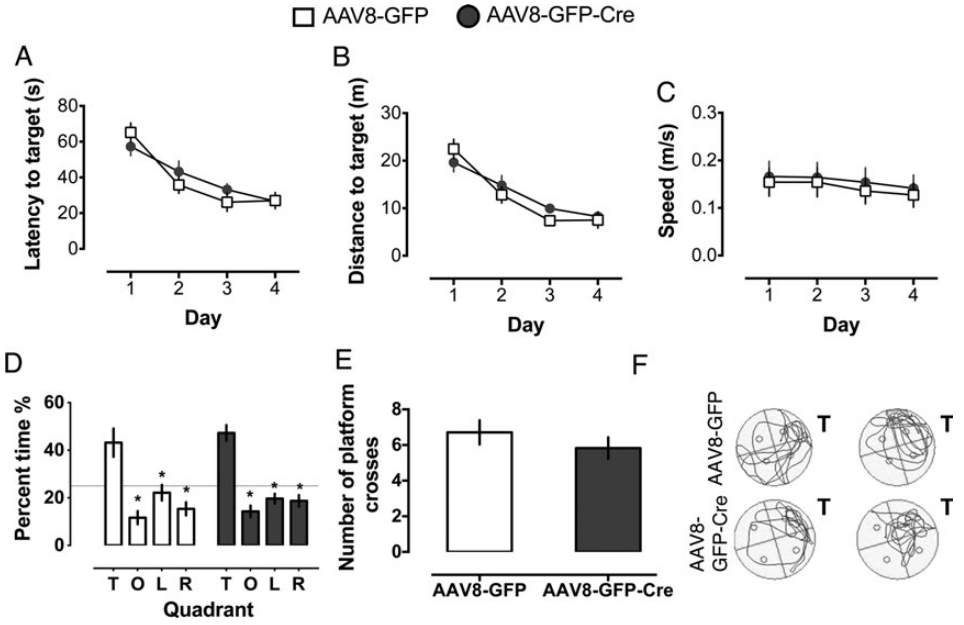


Figure 4. Performance of medial septum AAV8-GFP-Cre-injected mice in the MWM. VAChT$^{flox/flox}$ injected with AAV8-GFP virus (clear squares, $n = 14$) or AAV8-GFP-Cre virus (dark circles, $n = 14$) were tested in the spatial paradigm of the MWM. Data average of four 90-s trials per day were plotted. (*A*) Latency to reach the platform, (*B*) distance to reach the platform, (*C*) speed to reach the platform, (*D*) the percentage of time spent in each quadrant of the pool measured on Day 5 in a 60-s probe trial with the platform removed. (*E*) Number of platform crosses during the probe trial. (*F*) Representative path traces of 2 AAV8-GFP and 2 AAV8-GFP-Cre-injected mice in the probe trial. The target quadrant is in the upper right. Data are mean ± SEM. *$P < 0.05$. T, target; O, opposite; L, left; R, right.

no interaction $F_{3,30} = 1.501$, $P = 0.2342$, Fig. 5A–C). Notably, on the probe trial, control mice spent considerably more time in the target quadrant compared with the other quadrants (2-way ANOVA shows a significant effect of quadrant, $F_{3,80} = 7.226$, $P = 0.0002$, and an interaction effect $F_{3,80} = 3.133$, $P = 0.0301$, Fig. 5D), while VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice visited all quadrants almost equally.

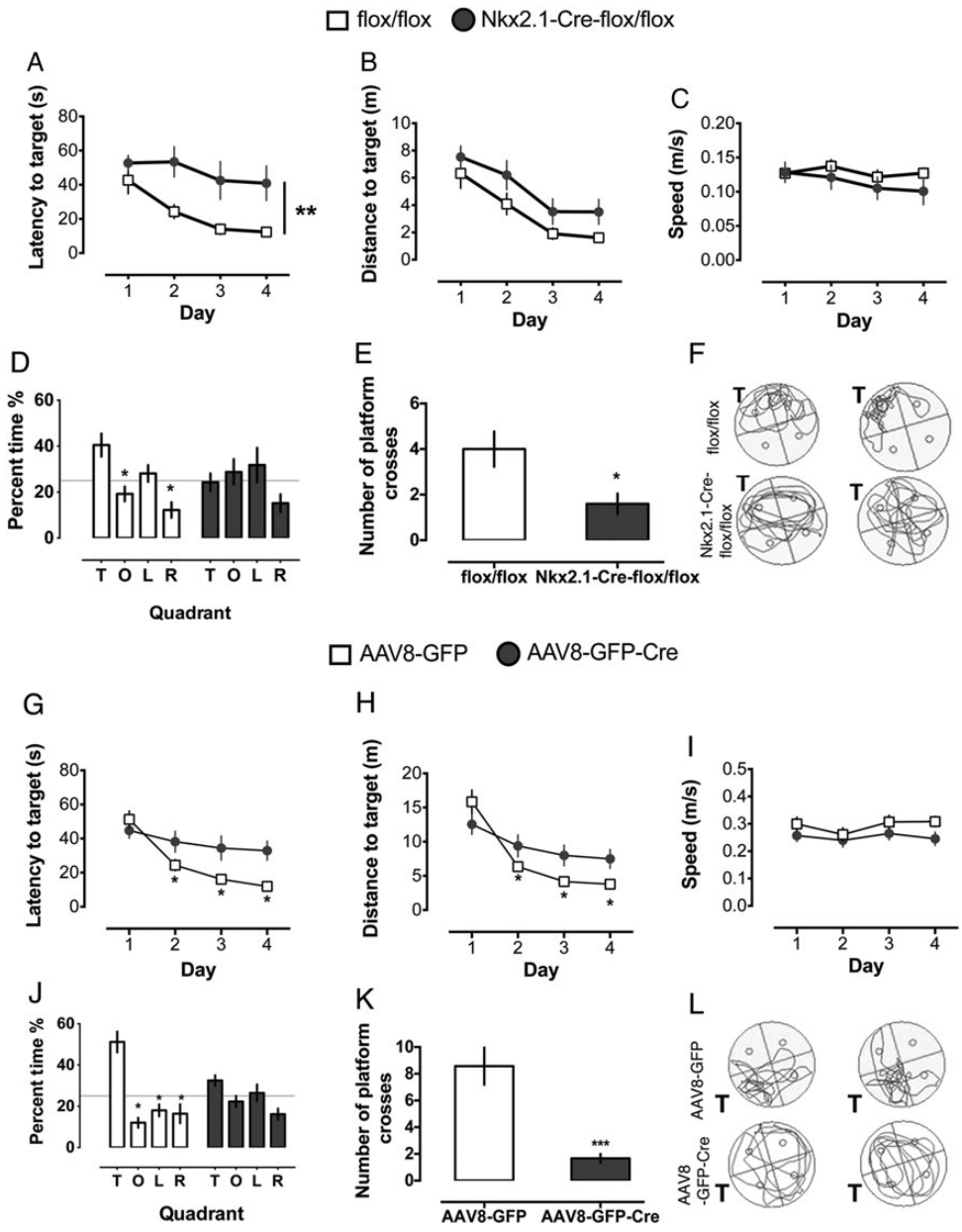


Figure 5. Reversal learning is affected in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ and medial septum AAV8-GFP-Cre-injected mice. VAChT$^{flox/flox}$ (clear squares, $n = 11$) and VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ (dark circles, $n = 11$) were tested in the reversal paradigm of the MWM. Data average of four 90-s trials per day were plotted. (*A*) Latency to reach the platform, (*B*) distance to reach the platform, (*C*) speed to reach the platform, (*D*) the percentage of time spent in each quadrant of the pool measured on Day 5 in a 60-s probe trial with the platform removed. (*E*) Number of platform crosses during the probe trial. (*F*) Representative path traces for 2 VAChT$^{flox/flox}$ and 2 VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ in the probe trial. The target quadrant is in the upper left. (*G*–*L*) AAV8-GFP (clear squares, $n = 14$) or AAV8-GFP-Cre (dark circles, $n = 14$)-injected mice were tested in the reversal paradigm of the MWM. The data average of four 90-s trials per day were plotted. (*G*) Latency to find the platform, (*H*) distance, (*I*) speed, (*J*) the percentage of time spent in each quadrant of the pool was measured on Day 5 in a 60-s probe trial with the platform removed. (*K*) Number of platform crosses during the probe trial. (*L*) Two AAV8-GFP- and 2 AAV8-GFP-Cre-injected mice in the probe trial. The target quadrant is indicated with a T. Data are mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$. T, target; O, opposite; L, left; R, right.

The number of platform crosses was also higher for control mice compared with VAChT mutants ($t_{(20)} = 2.797$, $P = 0.0111$, Fig. 5*E*). These results indicate that, different from control mice, VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice were unable to extinguish the previously learned position and relearn the new position of the hidden platform.

To account for compromised striatal cholinergic signaling in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice for the performance in the MWM (see Supplementary Fig. 1*D*), we also tested a mouse line with selective deletion of VAChT in striatal neurons (VAChT$^{D2\text{-}Cre\text{-}flox/flox}$), but spared hippocampal VAChT (Guzman et al. 2011; see Supplementary Fig. 7). Interestingly, VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice did not differ from controls (VAChT$^{flox/flox}$) in both acquisition and reversal versions on the MWM (see Supplementary Fig. 7*D*–*H*). These results suggest that deficits seen in reversal learning in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice are not likely due to impaired striatal cholinergic transmission, but rather a result of hippocampal or cortical deficits or combined cortical hippocampal

dysfunction. To discern among these possibilities, we used virus-injected mice.

Selective reduction of hippocampal cholinergic tone in virus-injected mice also increased latency to find the platform in reversal learning (2-way RM ANOVA shows an effect of days, $F_{3,39} = 21.96$, $P < 0.0001$ and a significant interaction effect, $F_{3,39} = 7.507$, $P = 0.0004$), with *post hoc* analysis revealing that AAV8-GFP-Cre-injected mice performed significantly worse on Day 4 compared with controls (Fig. 5G). During the probe trial, mice injected with AAV8-GFP-Cre virus showed significant impairments, failing to show a preference for the target quadrant (2-way ANOVA shows a significant effect of quadrant, $F_{3,104} = 23.3$, $P < 0.0001$, and an interaction effect, $F_{3,104} = 7.173$, $P = 0.002$, Fig. 5J). *Post hoc* analysis revealed that the AAV8-GFP-Cre mice did not prefer the target quadrant compared with the other quadrants, while the AAV8-GFP-injected controls had a strong preference for the target quadrant. Furthermore, the AAV8-GFP-Cre-injected mice showed a decrease in the number of platform crosses ($t_{(26)} = 0.9547$, $P = 0.0010$, Fig. 5K). These results reveal that disruption of hippocampal cholinergic tone, but not striatal or cortical cholinergic activity, compromises information processing in the MWM reversal learning.

Regulation of Working Memory by Septohippocampal VAChT

To determine whether other cognitive domains of importance in neuropsychiatric disorders that could contribute to the PAL deficits may also be regulated by synaptically released ACh, we evaluated the performance of the VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice on 2 measures of working memory: the working memory version of the MWM and spontaneous alternations in the Y-maze. In the working memory version of the MWM, VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice failed to improve their performance from the first to the second trial resulting in significant impairments in measures of latency savings ($t_{(12)} = 3.580$, $P = 0.0030$, Fig. 6A) and distance savings ($t_{(12)} = 2.852$, $P = 0.0127$, Fig. 6B), suggesting that the VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice have impaired working memory. Similarly, VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice revisited arms in the maze more often than controls resulting in a significant decrease in spontaneous alternations in the Y-maze ($t_{(12)} = 2.674$, $P = 0.0182$, Fig. 6C), suggesting that forebrain VAChT is required for normal working memory performance.

When tested on the working memory MWM test, mice with selective elimination of septohippocampal VAChT by virus injection (same cohort used in the MWM) also showed impaired latency savings ratio ($t_{(26)} = 2.847$, $P = 0.0111$, Fig. 6D) and distance savings ratio ($t_{(26)} = 2.149$, $P = 0.0473$, Fig. 6E). On the spontaneous alternations Y-maze task, AAV8-GFP-Cre-injected mice showed impairments on working memory, measured as a significant decreased rate of spontaneous alternations ($t_{(26)} = 3.347$, $P = 0.0041$, Fig. 6F). It is interesting to note that working memory deficits observed for AAV8-GFP-Cre-injected mice were similar to deficits observed for VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice. Taken together these results indicate that working memory is highly sensitive to hippocampal cholinergic tone.

Discussion

The present work shows that selective inhibition of cholinergic signaling in the hippocampus in mice leads to disruption of synaptic plasticity and specific cognitive impairments. In particular, we show that hippocampal cholinergic signaling is important for the modulation of cognitive tasks shown to be impaired in schizophrenia and dementia, including the PAL task. Interestingly, some hippocampal-dependent tasks appear to be more sensitive to decreased cholinergic signaling than others. Our results provide a comprehensive map of cholinergic-regulated hippocampal cognitive processing that may be useful to understand similar deficits in humans with cholinergic deficiency.

Notably, we report novel data indicating the importance of cholinergic signaling in regulating the PAL task. Clinically, the PAL task has been suggested as a potential cognitive marker of decline in psychosis (Wood et al. 2002). Significant impairments

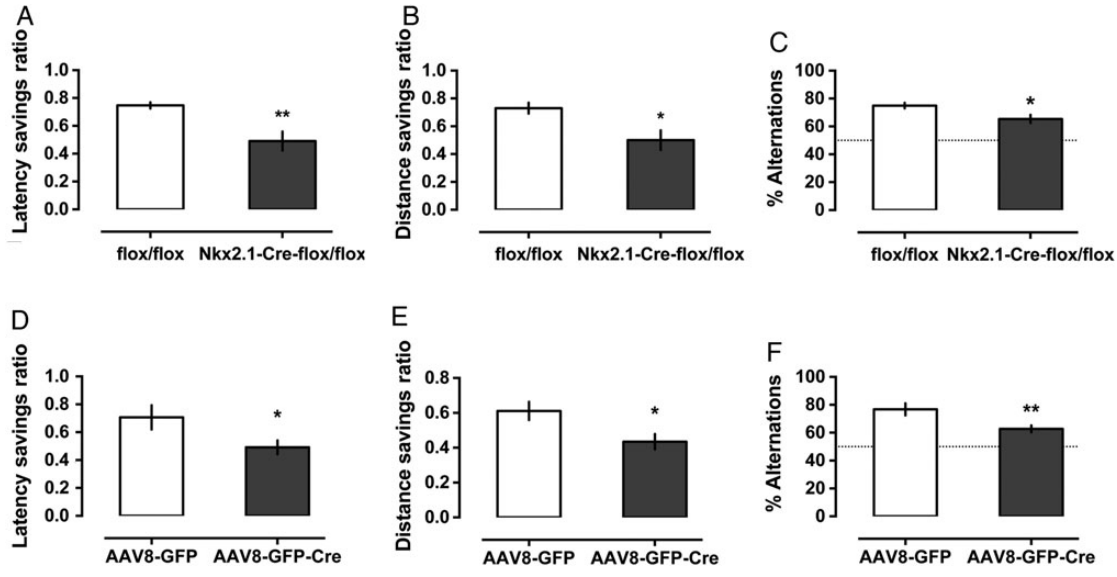


Figure 6. Working memory depends on hippocampal cholinergic tone. (*A*) Latency savings ratio and (*B*) distance savings ratio for VAChT$^{flox/flox}$ (clear, $n = 7$) and VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ (dark, $n = 7$) mice in the working memory version of the MWM. (*C*) Spontaneous alternations in the Y-maze for VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$. (*D*) Latency savings ratio and (*E*) distance savings ratio for AAV8-GFP (clear, $n = 14$) and AAV8-GFP-Cre (dark, $n = 14$) mice in the working memory version of the MWM. (*F*) Spontaneous alternations in the Y-maze for virus-injected mice. Data are mean ± SEM. *$P < 0.05$, **$P < 0.01$.

in PAL have been observed in patients with schizophrenia with a positive correlation between failure on the PAL task and negative symptoms (Barnett et al. 2005). Additionally, hippocampal activation during PAL is changed in patients with mild cognitive impairment compared with aged-matched controls (de Rover et al. 2011). Hence, PAL has also been considered a sensitive task for predicting cognitive decline in AD (Swainson et al. 2001; Blackwell et al. 2004).

Nonetheless, whether cholinergic signaling is required for acquisition of the task has not been clearly established. Systemic administration of donepezil, a cholinesterase inhibitor, improved post-acquisition PAL performance in mice, an effect that was attenuated with administration of muscarinic antagonists (Bartko, Vendrell et al. 2011). Similar results have been observed in monkeys where both mecamylamine (nicotinic antagonist) and scopolamine (muscarinic antagonist) induced deficits in PAL performance (Taffe et al. 2002; Katner et al. 2004). These results suggest that cholinergic signaling might be relevant for PAL. Also, rats previously trained in PAL that received injections into the dorsal hippocampus of either scopolamine or mecamylamine and that were re-tested did not show deficits in performance, suggesting that hippocampal cholinergic signaling might not modulate recall in this task (Talpos et al. 2009). Our results indicate that disruption in forebrain cholinergic tone disturbs PAL learning. Additionally, our data suggest that dysfunctional hippocampal cholinergic signaling may decrease PAL performance, as performance of mice in the PAL task correlates with hippocampal VAChT protein levels. Importantly, these deficits occurred in the absence of alterations in latency to touch the screen or to collect the reward, indicating that motivation was not a factor in the poorer performance of mice with lower cholinergic tone. Interestingly, mice deficient for the M1 receptor presented no differences compared with controls in their acquisition of the PAL task (Bartko, Romberg et al. 2011), suggesting that nicotinic and/or other muscarinic receptors might be involved in mediating learning in this hippocampal-dependent task.

Performance in PAL, as well as in other paired-associated tasks, may depend on intact hippocampal function in humans and rats (Talpos et al. 2009; de Rover et al. 2011). For example, short-lasting inactivation of the rat hippocampus using lidocaine (non-selective Na+ channel blocker) significantly impairs performance postacquisition of the PAL task, suggesting that the hippocampus is required at least for performance in this task (Talpos et al. 2009). In addition, human fMRI studies have shown bilateral BOLD activation of the hippocampus during the encoding phase of the PAL task (de Rover et al. 2011). Interestingly, subjects with memory deficits showed decreased hippocampal activation with increased memory demand, whereas healthy controls showed the opposite (de Rover et al. 2011). Moreover, PAL performance correlates with hippocampal volume loss in schizophrenia and mild cognitive impairment (MCI) (Keri et al. 2012). Intriguingly, recent reports indicate that mice with hippocampal lesions are still able to acquire the PAL task (Delotterie et al. 2015; Kim et al. 2015). One possible explanation (Kim et al. 2015) regarding these findings is that with a functional hippocampus the task is acquired in a hippocampal-dependent manner, but with a dysfunctional hippocampus, the task can be learnt using an alternative hippocampal-independent strategy. For example, mice with hippocampal lesions could have used the dorsal striatum to acquire the task (Delotterie et al. 2015). Indeed, the development of such behavioral plasticity has been shown in rats with unilateral hippocampal lesions (Zou et al. 1999). Our findings that forebrain VAChT-deficient mice seem unable to acquire the task, whereas decreased VAChT levels in the hippocampus decrease acquisition performance, suggest that the hippocampal cholinergic tone may facilitate acquisition of the PAL task. However, it is unlikely that only one brain region is involved in such a complex task.

The mechanisms by which ACh tone facilitates PAL performance are not fully understood. It is possible that cholinergic tone is required for specific types of synaptic plasticity. Indeed, hippocampal LTP in vitro is disturbed in a different mouse line lacking forebrain VAChT (Martyn et al. 2012). We corroborated this finding in vivo in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice and demonstrated that in the absence of VAChT expression, hippocampal LTP is compromised, suggesting that disturbances of synaptic plasticity might contribute to the deficit. To note, previous studies have shown that levels of VAChT are correlated to levels of ACh release (Prado et al. 2006; de Castro, Pereira, et al. 2009, reviewed in Prado et al. (2013)); an increase in VAChT levels increases ACh release whereas decreased levels have the opposite effect (Song et al. 1997; Prado et al. 2006; Kolisnyk, Guzman, et al. 2013). VAChT is decreased in AD (Parent et al. 2013). These results suggest that correlating levels of VAChT detected with PET ligands (Efange 2000) to performance in the PAL test (Harel et al. 2013) might provide a potential biomarker of remaining cholinergic function and cognitive reserve.

We showed that acquisition of the spatial version of the MWM and recall of platform location was mildly affected in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice, while AAV8-GFP-Cre-injected mice did not show any deficit in this behavioral task. Similarly, impairments in the spatial version of the MWM have been observed in rats with combined lesions of MS/VDB and nucleus basalis magnocellularis (NBM) cholinergic neurons produced by immunotoxin 192 IgG-saporin (Pizzo et al. 2002), while rats with immunotoxin lesions restricted to MS/VDB did not show any impairment (Berger-Sweeney et al. 1994; Baxter and Gallagher 1996; Pizzo et al. 2002; Frick et al. 2004). Interestingly, rats with 192 IgG-saporin lesions restricted to NBM also did not show behavioral impairments in the MWM (Pizzo et al. 2002). These data suggest that forebrain cholinergic signaling is necessary for reference spatial learning and memory assessed using the MWM; however, it seems that both the cortical and hippocampal cholinergic projections need to be compromised to produce a severe spatial deficit. Thus, providing that cortical cholinergic projections are intact, hippocampal cholinergic activity is not absolutely required for this behavioral task. It remains to be established whether GABA or glutamate, which could potentially be co-released with ACh (Guzman et al. 2011; Saunders et al. 2015) in both the hippocampus and cortex, may contribute to regulation of spatial memory by cholinergic neurons.

In contrast to the reference memory test, both VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ and AAV8-GFP-Cre-GFP-injected mice when tested in the MWM reversal learning task presented extensive deficits, suggesting a prominent role for hippocampal cholinergic signaling in reversal learning. The impairments seen in VAChT-deficient mice in reversal learning could relate to the loss of muscarinic presynaptic inhibition of excitatory feedback within cortical circuits (Hasselmo and McGaughy 2004), which would slow the extinction of a previously learned strategy (Hasselmo et al. 2002; Hasselmo 2006). To note, the findings with VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice recapitulated the deficits seen in reversal learning in a different mouse line with deficient forebrain cholinergic tone we generated previously (Martyn et al. 2012; Kolisnyk, Al-Onaizi, et al. 2013). Interestingly, rats with 192 IgG-saporin lesions restricted to NBM also show behavioral flexibility impairments (Cabrera et al. 2006). Taken together, these results suggest that both NBM-cortical and septohippocampal cholinergic

signaling might be critical for the mediation of this form of cognitive flexibility.

The most common form of LTP underlying hippocampal synaptic plasticity in spatial memory depends on the activation of NMDARs (Collingridge et al. 1983; Martin et al. 2000; MacDonald et al. 2006). Intracerebroventricular administration of a NMDAR antagonist (AP5) significantly impaired performance of rats during reversal testing in the MWM (Morris et al. 1990). Moreover, genetically modified mice with deletion of the GluN2B subunit of NMDARs in the CA1 region of the hippocampus exhibited impairments in reversal learning (von Engelhardt et al. 2008). Similarly, mice with corticohippocampal deletion of GluN2B present deficits in hippocampal synaptic plasticity, highlighted by abolished long-term depression (LTD), a partial deficiency of LTP, and memory impairments (Brigman et al. 2010). The impairments observed in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice in LTP and reversal learning suggest that long-term cholinergic signaling may regulate NMDAR-mediated synaptic plasticity required for reversal learning in the MWM.

Both prefrontal cortex and hippocampus have been implicated in working memory (Yoon et al. 2008). A number of studies indicate that cholinergic neurotransmission is crucial for modulation of working memory in various behavioral tasks (Levy et al. 1991; Baxter et al. 1995; Furey et al. 2000; Hironaka et al. 2001). Whether cholinergic modulation of working memory is dependent on ACh acting on prefrontal cortex, hippocampus, or in both structures simultaneously is not known. Our results show that deficits in the working memory version of the MWM task and the Y-maze alternating task are equally severe in both forebrain VAChT mutants (VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice) and hippocampus VAChT mutants (AAV8-GFP-Cre-injected mice), suggesting that hippocampal cholinergic tone is vital in regulating information processing in working memory tasks. Taken together, these results suggest that ACh may exert important roles in working memory via modulation of hippocampal function. Whether these working memory deficits somehow contribute to the poor performance in PAL remains to be established.

Imaging studies involving volumetric measurement of basal forebrain cholinergic nuclei in humans reveal a drastic decrease in the volume of basal forebrain neurons in AD and MCI patients, in comparison to healthy elderly controls (Grothe et al. 2010, 2012; Grothe, Ewers, et al. 2014; Teipel et al. 2014). Given that individuals with dementia may present long-term changes in cholinergic tone, our mouse lines and approaches may be directly relevant to understand molecular, cellular, circuitry, and behavioral consequences of cholinergic malfunction. The present work is relevant to understand how drug-induced cholinergic dysfunction or degenerative changes in cholinergic neurons contribute to cognitive alterations in several neuropsychiatric disorders (Severance and Yolken 2008; Scarr et al. 2009). In summary, hippocampal cholinergic activity does not seem to be critical for spatial reference learning and memory, but has fundamental roles on working memory, reversal learning, and paired associates learning. As PAL performance may be dependent on cholinergic integrity, it is tempting to speculate that the PAL task could be used to identify individuals with cognitive dysfunction linked to cholinergic abnormalities.

Supplementary Material

Supplementary material can be found at http://www.cercor.oxfordjournals.org/ online.

Funding

This work was supported by CIHR (MOP 93651, 12600, 89919), NSERC (402524-2013), the Weston Brain Institute, Brain Canada, Canadian Foundation for Innovation, ORF (Ontario research Fund), fellowship support from Kuwait University to M.A.A-O, the Annie Dakens Research Fund Award from the Alzheimer's Society fellowship to B.K. and a fellowship from the Heart and Stroke Foundation of Canada to M.S.G. G.M.P. received a graduate fellowship and D.M.B. is a research fellow (304389/2012-9) from the Brazilian National Council for Scientific and Technological Development (CNPq).

Notes

The authors declare no competing financial interests. We thank Dr Ashbeel Roy (The University of Western Ontario) for proofreading this manuscript and Dr R. Jane Rylett for a gift of antibodies. *Conflict of Interest*: None declared.

References

Anagnostaras SG, Maren S, Sage JR, Goodrich S, Fanselow MS. 1999. Scopolamine and Pavlovian fear conditioning in rats: dose-effect analysis. Neuropsychopharmacology. 21:731–744.

Anagnostaras SG, Murphy GG, Hamilton SE, Mitchell SL, Rahnama NP, Nathanson NM, Silva AJ. 2003. Selective cognitive dysfunction in acetylcholine M1 muscarinic receptor mutant mice. Nat Neurosci. 6:51–58.

Barnett JH, Sahakian BJ, Werners U, Hill KE, Brazil R, Gallagher O, Bullmore ET, Jones PB. 2005. Visuospatial learning and executive function are independently impaired in first-episode psychosis. Psychol Med. 35:1031–1041.

Bartko SJ, Romberg C, White B, Wess J, Bussey TJ, Saksida LM. 2011. Intact attentional processing but abnormal responding in M1 muscarinic receptor-deficient mice using an automated touchscreen method. Neuropharmacology. 61:1366–1378.

Bartko SJ, Vendrell I, Saksida LM, Bussey TJ. 2011. A computer-automated touchscreen paired-associates learning (PAL) task for mice: impairments following administration of scopolamine or dicyclomine and improvements following donepezil. Psychopharmacology (Berl). 214:537–548.

Bartus RT. 2000. On neurodegenerative diseases, models, and treatment strategies: lessons learned and lessons forgotten a generation following the cholinergic hypothesis. Exp Neurol. 163:495–529.

Baxter MG, Bucci DJ. 2013. Selective immunotoxic lesions of basal forebrain cholinergic neurons: twenty years of research and new directions. Behav Neurosci. 127:611–618.

Baxter MG, Bucci DJ, Gorman LK, Wiley RG, Gallagher M. 1995. Selective immunotoxic lesions of basal forebrain cholinergic cells: effects on learning and memory in rats. Behav Neurosci. 109:714–722.

Baxter MG, Gallagher M. 1996. Intact spatial learning in both young and aged rats following selective removal of hippocampal cholinergic input. Behav Neurosci. 110:460–467.

Berger-Sweeney J, Heckers S, Mesulam MM, Wiley RG, Lappi DA, Sharma M. 1994. Differential effects on spatial navigation of immunotoxin-induced cholinergic lesions of the medial septal area and nucleus basalis magnocellularis. J Neurosci. 14:4507–4519.

Blackwell AD, Sahakian BJ, Vesey R, Semple JM, Robbins TW, Hodges JR. 2004. Detecting dementia: novel neuropsychological

markers of preclinical Alzheimer's disease. Dement Geriatr Cogn Disord. 17:42–48.
Brigman JL, Wright T, Talani G, Prasad-Mulcare S, Jinde S, Seabold GK, Mathur P, Davis MI, Bock R, Gustin RM, et al. 2010. Loss of GluN2B-containing NMDA receptors in CA1 hippocampus and cortex impairs long-term depression, reduces dendritic spine density, and disrupts learning. J Neurosci. 30:4590–4600.
Cabrera SM, Chavez CM, Corley SR, Kitto MR, Butt AE. 2006. Selective lesions of the nucleus basalis magnocellularis impair cognitive flexibility. Behav Neurosci. 120:298–306.
Chudasama Y, Dalley JW, Nathwani F, Bouger P, Robbins TW. 2004. Cholinergic modulation of visual attention and working memory: dissociable effects of basal forebrain 192-IgG-saporin lesions and intraprefrontal infusions of scopolamine. Learn Mem. 11:78–86.
Collingridge GL, Kehl SJ, McLennan H. 1983. Excitatory amino acids in synaptic transmission in the Schaffer collateral-commissural pathway of the rat hippocampus. J Physiol. 334:33–46.
de Castro BM, De Jaeger X, Martins-Silva C, Lima RD, Amaral E, Menezes C, Lima P, Neves CM, Pires RG, Gould TW, et al. 2009. The vesicular acetylcholine transporter is required for neuromuscular development and function. Mol Cell Biol. 29:5238–5250.
de Castro BM, Pereira GS, Magalhaes V, Rossato JI, De Jaeger X, Martins-Silva C, Leles B, Lima P, Gomez MV, Gainetdinov RR, et al. 2009. Reduced expression of the vesicular acetylcholine transporter causes learning deficits in mice. Genes Brain Behav. 8:23–35.
de Rover M, Pironti VA, McCabe JA, Acosta-Cabronero J, Arana FS, Morein-Zamir S, Hodges JR, Robbins TW, Fletcher PC, Nestor PJ, et al. 2011. Hippocampal dysfunction in patients with mild cognitive impairment: a functional neuroimaging study of a visuospatial paired associates learning task. Neuropsychologia. 49:2060–2070.
Decker MW, Majchrzak MJ. 1992. Effects of systemic and intracerebroventricular administration of mecamylamine, a nicotinic cholinergic antagonist, on spatial memory in rats. Psychopharmacology (Berl). 107:530–534.
Delotterie DF, Mathis C, Cassel JC, Rosenbrock H, Dorner-Ciossek C, Marti A. 2015. Touchscreen tasks in mice to demonstrate differences between hippocampal and striatal functions. Neurobiol Learn Mem. 120:16–27.
Dennis SH, Pasqui F, Colvin EM, Sanger H, Mogg AJ, Felder CC, Broad LM, Fitzjohn SM, Isaac JT, Mellor JR. 2015. Activation of muscarinic M1 acetylcholine receptors induces long-term potentiation in the hippocampus. Cereb Cortex. 26:414–426.
Efange SM. 2000. In vivo imaging of the vesicular acetylcholine transporter and the vesicular monoamine transporter. FASEB J. 14:2401–2413.
El Mestikawy S, Wallen-Mackenzie A, Fortin GM, Descarries L, Trudeau LE. 2011. From glutamate co-release to vesicular synergy: vesicular glutamate transporters. Nat Rev Neurosci. 12:204–216.
Franklin KBJ, Paxinos G. 2008. The mouse brain in stereotaxic coordinates. Amsterdam (NY): Elsevier/Academic Press.
Frick KM, Kim JJ, Baxter MG. 2004. Effects of complete immunotoxin lesions of the cholinergic basal forebrain on fear conditioning and spatial learning. Hippocampus. 14:244–254.
Furey ML, Pietrini P, Haxby JV. 2000. Cholinergic enhancement and increased selectivity of perceptual processing during working memory. Science. 290:2315–2319.
Gale GD, Anagnostaras SG, Fanselow MS. 2001. Cholinergic modulation of pavlovian fear conditioning: effects of intrahippocampal scopolamine infusion. Hippocampus. 11:371–376.
Gautam D, Duttaroy A, Cui Y, Han SJ, Deng C, Seeger T, Alzheimer C, Wess J. 2006. M1-M3 muscarinic acetylcholine receptor-deficient mice: novel phenotypes. J Mol Neurosci. 30:157–160.
Ge S, Dani JA. 2005. Nicotinic acetylcholine receptors at glutamate synapses facilitate long-term depression or potentiation. J Neurosci. 25:6084–6091.
Giessel AJ, Sabatini BL. 2010. M1 muscarinic receptors boost synaptic potentials and calcium influx in dendritic spines by inhibiting postsynaptic SK channels. Neuron. 68:936–947.
Gil-Bea FJ, Solas M, Mateos L, Winblad B, Ramirez MJ, Cedazo-Minguez A. 2011. Cholinergic hypofunction impairs memory acquisition possibly through hippocampal Arc and BDNF downregulation. Hippocampus. 21:999–1009.
Gras C, Amilhon B, Lepicard EM, Poirel O, Vinatier J, Herbin M, Dumas S, Tzavara ET, Wade MR, Nomikos GG, et al. 2008. The vesicular glutamate transporter VGLUT3 synergizes striatal acetylcholine tone. Nat Neurosci. 11:292–300.
Gray R, Rajan AS, Radcliffe KA, Yakehiro M, Dani JA. 1996. Hippocampal synaptic transmission enhanced by low concentrations of nicotine. Nature. 383:713–716.
Gray SL, Anderson ML, Dublin S, Hanlon JT, Hubbard R, Walker R, Yu O, Crane PK, Larson EB. 2015. Cumulative use of strong anticholinergics and incident dementia: a prospective cohort study. JAMA Intern Med. 175:401–407.
Grothe M, Heinsen H, Teipel SJ. 2012. Atrophy of the cholinergic basal forebrain over the adult age range and in early stages of Alzheimer's disease. Biol Psychiatry. 71:805–813.
Grothe M, Zaborszky L, Atienza M, Gil-Neciga E, Rodriguez-Romero R, Teipel SJ, Amunts K, Suarez-Gonzalez A, Cantero JL. 2010. Reduction of basal forebrain cholinergic system parallels cognitive impairment in patients at high risk of developing Alzheimer's disease. Cereb Cortex. 20:1685–1695.
Grothe MJ, Ewers M, Krause B, Heinsen H, Teipel SJ, Alzheimer's Disease Neuroimaging I. 2014. Basal forebrain atrophy and cortical amyloid deposition in nondemented elderly subjects. Alzheimer's Dementia. 10:S344–S353.
Grothe MJ, Schuster C, Bauer F, Heinsen H, Prudlo J, Teipel SJ. 2014. Atrophy of the cholinergic basal forebrain in dementia with Lewy bodies and Alzheimer's disease dementia. J Neurol. 261:1939–1948.
Gu Z, Yakel JL. 2011. Timing-dependent septal cholinergic induction of dynamic hippocampal synaptic plasticity. Neuron. 71:155–165.
Guzman MS, De Jaeger X, Raulic S, Souza IA, Li AX, Schmid S, Menon RS, Gainetdinov RR, Caron MG, Bartha R, et al. 2011. Elimination of the vesicular acetylcholine transporter in the striatum reveals regulation of behaviour by cholinergic-glutamatergic co-transmission. PLoS Biol. 9:e1001194.
Harel BT, Pietrzak RH, Snyder PJ, Maruff P. 2013. Effect of cholinergic neurotransmission modulation on visual spatial paired associate learning in healthy human adults. Psychopharmacology (Berl). 228:673–683.
Hasselmo ME. 2006. The role of acetylcholine in learning and memory. Curr Opin Neurobiol. 16:710–715.
Hasselmo ME, Bodelon C, Wyble BP. 2002. A proposed function for hippocampal theta rhythm: separate phases of encoding and retrieval enhance reversal of prior learning. Neural Comput. 14:793–817.
Hasselmo ME, McGaughy J. 2004. High acetylcholine levels set circuit dynamics for attention and encoding and low acetylcholine levels set dynamics for consolidation. Prog Brain Res. 145:207–231.

Hironaka N, Tanaka K, Izaki Y, Hori K, Nomura M. 2001. Memory-related acetylcholine efflux from rat prefrontal cortex and hippocampus: a microdialysis study. Brain Res. 901:143–150.

Hutchison RM, Chidiac P, Leung LS. 2009. Hippocampal long-term potentiation is enhanced in urethane-anesthetized RGS2 knockout mice. Hippocampus. 19:687–691.

Janis LS, Glasier MM, Fulop Z, Stein DG. 1998. Intraseptal injections of 192 IgG saporin produce deficits for strategy selection in spatial-memory tasks. Behav Brain Res. 90:23–34.

Ji D, Dani JA. 2000. Inhibition and disinhibition of pyramidal neurons by activation of nicotinic receptors on hippocampal interneurons. J Neurophysiol. 83:2682–2690.

Ji D, Lape R, Dani JA. 2001. Timing and location of nicotinic activity enhances or depresses hippocampal synaptic plasticity. Neuron. 31:131–141.

Katner SN, Davis SA, Kirsten AJ, Taffe MA. 2004. Effects of nicotine and mecamylamine on cognition in rhesus monkeys. Psychopharmacology (Berl). 175:225–240.

Keri S, Szamosi A, Benedek G, Kelemen O. 2012. How does the hippocampal formation mediate memory for stimuli processed by the magnocellular and parvocellular visual pathways? Evidence from the comparison of schizophrenia and amnestic mild cognitive impairment (aMCI). Neuropsychologia. 50:3193–3199.

Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. 2010. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol. 8:e1000412.

Kim CH, Heath CJ, Kent BA, Bussey TJ, Saksida LM. 2015. The role of the dorsal hippocampus in two versions of the touchscreen automated paired associates learning (PAL) task for mice. Psychopharmacology (Berl). 232:3899–3910.

Kitt CA, Hohmann C, Coyle JT, Price DL. 1994. Cholinergic innervation of mouse forebrain structures. J Comp Neurol. 341:117–129.

Kolisnyk B, Al-Onaizi MA, Hirata PH, Guzman MS, Nikolova S, Barbash S, Soreq H, Bartha R, Prado MA, Prado VF. 2013. Forebrain deletion of the vesicular acetylcholine transporter results in deficits in executive function, metabolic, and RNA splicing abnormalities in the prefrontal cortex. J Neurosci. 33:14908–14920.

Kolisnyk B, Guzman MS, Raulic S, Fan J, Magalhaes AC, Feng G, Gros R, Prado VF, Prado MA. 2013. ChAT-ChR2-EYFP mice have enhanced motor endurance but show deficits in attention and several additional cognitive domains. J Neurosci. 33:10427–10438.

Leung LS, Shen B, Rajakumar N, Ma J. 2003. Cholinergic activity enhances hippocampal long-term potentiation in CA1 during walking in rats. J Neurosci. 23:9297–9304.

Levy A, Kong RM, Stillman MJ, Shukitt-Hale B, Kadar T, Rauch TM, Lieberman HR. 1991. Nimodipine improves spatial working memory and elevates hippocampal acetylcholine in young rats. Pharmacol Biochem Behav. 39:781–786.

MacDonald JF, Jackson MF, Beazely MA. 2006. Hippocampal long-term synaptic plasticity and signal amplification of NMDA receptors. Crit Rev Neurobiol. 18:71–84.

Mar AC, Horner AE, Nilsson SR, Alsio J, Kent BA, Kim CH, Holmes A, Saksida LM, Bussey TJ. 2013. The touchscreen operant platform for assessing executive function in rats and mice. Nat Protoc. 8:1985–2005.

Martin SJ, Grimwood PD, Morris RG. 2000. Synaptic plasticity and memory: an evaluation of the hypothesis. Annu Rev Neurosci. 23:649–711.

Martins-Silva C, De Jaeger X, Guzman MS, Lima RD, Santos MS, Kushmerick C, Gomez MV, Caron MG, Prado MA, Prado VF. 2011. Novel strains of mice deficient for the vesicular acetylcholine transporter: insights on transcriptional regulation and control of locomotor behavior. PLoS ONE. 6:e17611.

Martyn AC, De Jaeger X, Magalhaes AC, Kesarwani R, Goncalves DF, Raulic S, Guzman MS, Jackson MF, Izquierdo I, Macdonald JF, et al. 2012. Elimination of the vesicular acetylcholine transporter in the forebrain causes hyperactivity and deficits in spatial memory and long-term potentiation. Proc Natl Acad Sci USA. 109:17651–17656.

Mesulam M. 2004. The cholinergic lesion of Alzheimer's disease: pivotal factor or side show? Learn Mem. 11:43–49.

Mesulam MM. 2013. Cholinergic circuitry of the human nucleus basalis and its fate in Alzheimer's disease. J Comp Neurol. 521:4124–4144.

Mesulam MM, Hersh LB, Mash DC, Geula C. 1992. Differential cholinergic innervation within functional subdivisions of the human cerebral cortex: a choline acetyltransferase study. J Comp Neurol. 318:316–328.

Moreau PH, Cosquer B, Jeltsch H, Cassel JC, Mathis C. 2008. Neuroanatomical and behavioral effects of a novel version of the cholinergic immunotoxin mu p75-saporin in mice. Hippocampus. 18:610–622.

Morris RG, Davis S, Butcher SP. 1990. Hippocampal synaptic plasticity and NMDA receptors: a role in information storage? Phil Trans R Soc Lond Ser B Biol Sci. 329:187–204.

Morton AJ, Skillings E, Bussey TJ, Saksida LM. 2006. Measuring cognitive deficits in disabled mice using an automated interactive touchscreen system. Nat Methods. 3:767.

Nelson AB, Bussert TG, Kreitzer AC, Seal RP. 2014. Striatal cholinergic neurotransmission requires VGLUT3. J Neurosci. 34:8772–8777.

Parent MB, Baxter MG. 2004. Septohippocampal acetylcholine: involved in but not necessary for learning and memory? Learn Mem. 11:9–20.

Parent MJ, Bedard MA, Aliaga A, Minuzzi L, Mechawar N, Soucy JP, Schirrmacher E, Kostikov A, Gauthier SG, Rosa-Neto P. 2013. Cholinergic depletion in Alzheimer's disease shown by [(18) F]FEOBV autoradiography. Int J Mol Imaging. 2013:205045.

Patel JC, Rossignol E, Rice ME, Machold RP. 2012. Opposing regulation of dopaminergic activity and exploratory motor behavior by forebrain and brainstem cholinergic circuits. Nat Commun. 3:1172.

Pichat P, Bergis OE, Terranova JP, Urani A, Duarte C, Santucci V, Gueudet C, Voltz C, Steinberg R, Stemmelin J, et al. 2007. SSR180711, a novel selective alpha7 nicotinic receptor partial agonist: (II) efficacy in experimental models predictive of activity against cognitive symptoms of schizophrenia. Neuropsychopharmacology. 32:17–34.

Pizzo DP, Thal LJ, Winkler J. 2002. Mnemonic deficits in animals depend upon the degree of cholinergic deficit and task complexity. Exp Neurol. 177:292–305.

Poulin B, Butcher A, McWilliams P, Bourgognon JM, Pawlak R, Kong KC, Bottrill A, Mistry S, Wess J, Rosethorne EM, et al. 2010. The M3-muscarinic receptor regulates learning and memory in a receptor phosphorylation/arrestin-dependent manner. Proc Natl Acad Sci USA. 107:9440–9445.

Prado VF, Martins-Silva C, de Castro BM, Lima RF, Barros DM, Amaral E, Ramsey AJ, Sotnikova TD, Ramirez MR, Kim HG, et al. 2006. Mice deficient for the vesicular acetylcholine transporter are myasthenic and have deficits in object and social recognition. Neuron. 51:601–612.

Prado VF, Roy A, Kolisnyk B, Gros R, Prado MA. 2013. Regulation of cholinergic activity by the vesicular acetylcholine transporter. Biochem J. 450:265–274.

Ragozzino ME, Artis S, Singh A, Twose TM, Beck JE, Messer WS Jr. 2012. The selective M1 muscarinic cholinergic agonist CDD-0102A enhances working memory and cognitive flexibility. J Pharmacol Exp Ther. 340:588–594.

Romberg C, Mattson MP, Mughal MR, Bussey TJ, Saksida LM. 2011. Impaired attention in the 3xTgAD mouse model of Alzheimer's disease: rescue by donepezil (Aricept). J Neurosci. 31:3500–3507.

Sango K, McDonald MP, Crawley JN, Mack ML, Tifft CJ, Skop E, Starr CM, Hoffmann A, Sandhoff K, Suzuki K, et al. 1996. Mice lacking both subunits of lysosomal beta-hexosaminidase display gangliosidosis and mucopolysaccharidosis. Nat Genet. 14:348–352.

Saunders A, Granger AJ, Sabatini BL. 2015. Corelease of acetylcholine and GABA from cholinergic forebrain neurons. eLife. 4: e06412.

Scarr E, Cowie TF, Kanellakis S, Sundram S, Pantelis C, Dean B. 2009. Decreased cortical muscarinic receptors define a subgroup of subjects with schizophrenia. Mol Psychiatry. 14:1017–1023.

Seeger T, Fedorova I, Zheng F, Miyakawa T, Koustova E, Gomeza J, Basile AS, Alzheimer C, Wess J. 2004. M2 muscarinic acetylcholine receptor knock-out mice show deficits in behavioral flexibility, working memory, and hippocampal plasticity. J Neurosci. 24:10117–10127.

Severance EG, Yolken RH. 2008. Novel alpha7 nicotinic receptor isoforms and deficient cholinergic transcription in schizophrenia. Genes Brain Behav. 7:37–45.

Song H, Ming G, Fon E, Bellocchio E, Edwards RH, Poo M. 1997. Expression of a putative vesicular acetylcholine transporter facilitates quantal transmitter packaging. Neuron. 18:815–826.

Swainson R, Hodges JR, Galton CJ, Semple J, Michael A, Dunn BD, Iddon JL, Robbins TW, Sahakian BJ. 2001. Early detection and differential diagnosis of Alzheimer's disease and depression with neuropsychological tasks. Dement Geriatr Cogn Disord. 12:265–280.

Taffe MA, Weed MR, Gutierrez T, Davis SA, Gold LH. 2002. Differential muscarinic and NMDA contributions to visuo-spatial paired-associate learning in rhesus monkeys. Psychopharmacology (Berl). 160:253–262.

Talpos JC, McTighe SM, Dias R, Saksida LM, Bussey TJ. 2010. Trial-unique, delayed nonmatching-to-location (TUNL): a novel, highly hippocampus-dependent automated touchscreen test of location memory and pattern separation. Neurobiol Learn Mem. 94:341–352.

Talpos JC, Winters BD, Dias R, Saksida LM, Bussey TJ. 2009. A novel touchscreen-automated paired-associate learning (PAL) task sensitive to pharmacological manipulation of the hippocampus: a translational rodent model of cognitive impairments in neurodegenerative disease. Psychopharmacology (Berl). 205:157–168.

Teipel S, Heinsen H, Amaro E Jr, Grinberg LT, Krause B, Grothe M, Alzheimer's Disease Neuroimaging Initiative. 2014. Cholinergic basal forebrain atrophy predicts amyloid burden in Alzheimer's disease. Neurobiol Aging. 35:482–491.

Timmermann DB, Gronlien JH, Kohlhaas KL, Nielsen EO, Dam E, Jorgensen TD, Ahring PK, Peters D, Holst D, Christensen JK, et al. 2007. An allosteric modulator of the alpha7 nicotinic acetylcholine receptor possessing cognition-enhancing properties in vivo. J Pharmacol Exp Ther. 323:294–307.

Vidal C, Changeux JP. 1993. Nicotinic and muscarinic modulations of excitatory synaptic transmission in the rat prefrontal cortex in vitro. Neuroscience. 56:23–32.

von Engelhardt J, Doganci B, Jensen V, Hvalby O, Gongrich C, Taylor A, Barkus C, Sanderson DJ, Rawlins JN, Seeburg PH, et al. 2008. Contribution of hippocampal and extra-hippocampal NR2B-containing NMDA receptors to performance on spatial learning tasks. Neuron. 60:846–860.

Vorhees CV, Williams MT. 2006. Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nat Protoc. 1:848–858.

Wallenstein GV, Vago DR. 2001. Intrahippocampal scopolamine impairs both acquisition and consolidation of contextual fear conditioning. Neurobiol Learn Mem. 75:245–252.

Walsh TJ, Herzog CD, Gandhi C, Stackman RW, Wiley RG. 1996. Injection of IgG 192-saporin into the medial septum produces cholinergic hypofunction and dose-dependent working memory deficits. Brain Res. 726:69–79.

Wood SJ, Proffitt T, Mahony K, Smith DJ, Buchanan JA, Brewer W, Stuart GW, Velakoulis D, McGorry PD, Pantelis C. 2002. Visuospatial memory and learning in first-episode schizophreniform psychosis and established schizophrenia: a functional correlate of hippocampal pathology? Psychol Med. 32:429–438.

Yoon T, Okada J, Jung MW, Kim JJ. 2008. Prefrontal cortex and hippocampus subserve different components of working memory in rats. Learn Mem. 15:97–105.

Zheng F, Wess J, Alzheimer C. 2012. M2 muscarinic acetylcholine receptors regulate long-term potentiation at hippocampal CA3 pyramidal cell synapses in an input-specific fashion. J Neurophysiol. 108:91–100.

Zoli M, Picciotto MR, Ferrari R, Cocchi D, Changeux JP. 1999. Increased neurodegeneration during ageing in mice lacking high-affinity nicotine receptors. EMBO J. 18:1235–1244.

Zou LB, Yamada K, Sasa M, Nabeshima T. 1999. Two phases of behavioral plasticity in rats following unilateral excitotoxic lesion of the hippocampus. Neuroscience. 92:819–826.

Curriculum Vitae

BENJAMIN KOLISNYK

Trainee, Robarts Research Institute

Graduate Program in Neuroscience, Schulich School of Medicine & Dentistry

EDUCATION:

UNIVERSITY	DEGREE	DEPARTMENT	YEAR
The University of Ottawa	**BSc. Hons**	**Psychology & Biochemistry**	**2008-12**
The University of Western Ontario	**PhD**	**Neuroscience**	**2012-Current**

Thesis Title: *Cholinergic Mechanisms Regulating Cognitive Function and RNA Metabolism*

Ph. D. Supervisor: Drs. Marco & Vania Prado

ACADEMIC EMPLOYMENT HISTORY:

TEACHING ASSISTANT

UNIVERSITY	SUPERVISOR	DEPARTMENT	YEAR
The University of Western Ontario	Dr. Michelle Belton	Medical Sciences	2013-Current
	Dr. Rhodri Cusak	Neuroscience	2014

AWARDS AND DISTINCTIONS

2014-2017	The Alzheimer Society Research Program Fellowship The Annie Dakens Research Fund Award
2015	ISN-APSN Travel Award
2012-2014	OGS Graduate Scholarship 2012-2013, 2013-2014
2012	CPA Certificate of Academic Excellence - Prize / Award Canadian Psychological Association for Best Thesis

PRESENTATIONS

Posters Presented

Kolisnyk B, Al-Onaizi MA, Hirata PH, Guzman MS, Nikolova S, Barbash S, Soreq H, Bartha R, Prado MA, Prado VF "Deletion of the vesicular acetylcholine transporter in the murine forebrain results in metabolic abnormalities in the prefrontal cortex and disruption in executive function.", Neuropsychopharmacology Meeting 2013, U.S.A, California, San Diego.

Kolisnyk B, Hirata PH, Prado MA, and Prado VF. "Cholinergic Tone And Attention: A Possible Role Of The a7 Nicotinic Acetylcholine Receptor", Canadian Neuroscience Meeting 2013, Canada, Ontario, Toronto

Kolisnyk, B, Al-Onaizi, M.A, Parfitt, G.M, Kish, M.T, Xu, J, Hanin,G, Soreq, H, Prado MA, and Prado VF "Cholinergic regulation of cognitive function and underlying molecular mechanisms", Canadian Neuroscience Meeting 2014, Canada , Quebec, Montreal **and invited to present at** the Canadian Student Health Research Forum 2014, Canada, Manitoba, Winnipeg.

Kolisnyk, B, Al-Onaizi, M.A, Prado MA, and Prado VF "Elucidating the role of the □7 nicotinic acetylcholine receptor in attention", Neurex Meeting: "Cholinergic Tone: Where from, what for?" 2014, France, Alsace, Strasbourg.

Kolisnyk, B, Al-Onaizi, M.A, Prado MA, and Prado VF "α7 nicotinic acetylcholine receptor-deficient mice exhibit sustained attention impairments that are reversed by ABT-418", ISN 2015 Biennial Meeting of the Society of Neurochemistry , Australia, Queensland, Cairns.

PUBLICATIONS

Journal Articles

Al-Onaizi, MA, Parfitt, GM, **Kolisnyk, B**, Law, CSH, Guzman, MS, Barros, DM, Leung, SL, Prado, MAM, Prado, VF “Regulation of cognitive processing by hippocampal cholinergic tone”. *Cerebral Cortex, piibhv349* (2016)

Beraldo FH, Thomas A, **Kolisnyk B**, Hirata PH, De Jaeger X, Martyn AC, Fan J, Goncalves DF, Cowan MF, Masood T, Martins VR, Gros R, Prado VF, Prado MA. Hyperactivity and attention deficits in mice with decreased levels of stress inducible phosphoprotein 1 (STIP1). Dis Model Mech. 2015 Sep 17

Kolisnyk B, Al-Onaizi MA, Prado VF, Prado MA. α7 nicotinic ACh receptor-deficient mice exhibit sustained attention impairments that are reversed by β2 nicotinic ACh receptor activation. Br J Pharmacol. 2015 Jul 29;

Nogueira Kde O, Sartori SS, Araújo VA, Neves CA, **Kolisnyk B**. (2015) Sperm storage in Hemidactylus mabouia: Morphological and ultrastructural aspects of a reproductive strategy. Anim Reprod Sci. 2015 Aug;159:212-6.

Kolisnyk B, Al-Onaizi MA, Hirata PH, Guzman MS, Nikolova S, Barbash S, Soreq H, Bartha R, Prado MA, Prado VF (2013) Forebrain deletion of the vesicular acetylcholine transporter results in deficits in executive function, metabolic, and RNA splicing abnormalities in the prefrontal cortex. *The Journal of Neuroscience* 33:14908-14920.

Kolisnyk, B., Guzman, M. S., Raulic, S., Fan, J., Magalhães, A. C., Feng, G., ... & Prado, M. A. (2013). ChAT–ChR2–EYFP Mice Have Enhanced Motor Endurance But Show Deficits in Attention and Several Additional Cognitive Domains. *The Journal of Neuroscience*, *33*(25), 10427-10438.

Prado, V.F., Roy, A., **Kolisnyk, B**, Gros, R. and Marco, M.A. "Regulation of cholinergic activity by the vesicular acetylcholine transporter." *Biochemical Journal* 450, no. 2 (2013): 265-274.

Kolisnyk, B, Al-Onaizi, MA, Soreq, L, Barbash S, Bekenstein, U, Haberman, N, Hanin, G, Kish, MT, Souza da Silva, J, Fahenstock, M, Ule, J, Soreq H, Prado, VF, Prado, MAM. "Cholinergic surveillance over hippocampal RNA metabolism and Alzheimer's-like pathology" Cerebral Cortex (2016)

Kolisnyk, B, Al-Onaizi, Xu, J, Parfitt, GM, Ostapchenko, VG, Hanin, G, Soreq H, Prado, MAM, Prado, VF. "Cholinergic Regulation of hnRNPA2/B1 Translation by M1 Muscarinic Receptors" *The Journal of Neuroscience (2016)*

References Available Upon Request