The Role of Forebrain Cholinergic Signalling In Regulating Hippocampal Function And Neuropathology

Mohammed Al-Onaizi
The University of Western Ontario

Supervisor
Dr. Vania F. Prado
The University of Western Ontario

Graduate Program in Anatomy and Cell Biology
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Abstract

Cholinergic dysfunction has been associated with cognitive abnormalities in a variety of neurodegenerative and neuropsychiatric disorders, including Alzheimer's Disease (AD). Cumulative use of drugs with anticholinergic activity is associated with increased risk for dementia and AD. Also, cholinergic function has been implicated in predicting the development of key neuropathological hallmarks seen in AD. However, the relationship between cholinergic dysfunction and conservation of cognitive ability as well as neuronal cell maintenance is not fully understood. Here, we tested how information processing and distinct molecular mechanisms associated with AD are regulated by cholinergic tone in genetically-modified mice in which cholinergic transmission was altered by targeting the vesicular acetylcholine transporter (VACht), a protein required for acetylcholine storage and release. We assessed the long-term consequences of loss of central cholinergic signalling for hippocampal vulnerability to age-induced stress. We show that deletion of forebrain-specific ACh release leads to age-related increases in neuronal vulnerability, protein aggregation, tau Thr-231 phosphorylation and misfolding, and neuroinflammation. Moreover, inhibition of forebrain cholinergic neurotransmission led to a disturbance in adult hippocampal neurogenesis, highlighted by decreased proliferation and cell survival in neural precursor cells. Additionally, 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 in the mouse PAL task was impaired in forebrain-specific VACht-
deficient mice, 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 signalling. In contrast, spatial memory was relatively preserved. Moreover, we assessed the functional consequence of impaired neurogenesis by testing pattern separation using a Location Discrimination task. Mice with compromised cholinergic signalling were impaired when stimuli were presented with small separation, but not when stimuli were presented with high separation, suggesting that deficient cholinergic tone has major consequences on pattern separation. The pathological changes in the hippocampus we observed in VACht-deficient mice have important consequences as they presented age-related deterioration in spatial navigation. Our findings provide a refined understanding of the importance of acetylcholine in modulating molecular mechanisms and key cognitive behaviours involved in AD.

**Keywords**

Acetylcholine  
Vesicular Acetylcholine Transporter  
Alzheimer's Disease  
Cognition  
Neurogenesis  
Pathology
Co-Authorship Statement

Mohammed A. Al-Onaizi performed all of the experiments in this thesis, except panels C-F in Figure 11, and Figure 15A under the supervision of Dr. Vania F. Prado and Dr. Marco A.M. Prado. Benjamin Kolisnyk performed Western blot analyses in panels C-F in Figure 11, and qPCR analyses Figure 15A under the supervision of Dr. Vania F. Prado and Dr. Marco A.M. Prado. The long-term potentiation experiment (Figure 22) to analyze hippocampal synaptic plasticity was performed in collaboration with Clayton S.H. Law under the supervision of Dr. Stan L. Leung (University of Western Ontario, Canada).
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<th>Full name</th>
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<tbody>
<tr>
<td>5-CSRTT</td>
<td>Five choice serial reaction time task</td>
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<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid beta</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
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<tr>
<td>C. elegans</td>
<td>Caenorhabditis elegans</td>
</tr>
<tr>
<td>EPSPs</td>
<td>Excitatory postsynaptic potentials</td>
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<tr>
<td>GPCRs</td>
<td>G-protein-coupled receptors</td>
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<tr>
<td>K⁺</td>
<td>Potassium</td>
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<tr>
<td>MAP</td>
<td>Microtubule-associated protein</td>
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<tr>
<td>MCI</td>
<td>Mild cognitive impairment</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
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<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>Na⁺</td>
<td>Sodium</td>
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<td>NBM</td>
<td>Nucleus basalis of Meynert</td>
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<td>NFT</td>
<td>Neurofibrillary tangles</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>p75NTR</td>
<td>p75 neurotrophin receptor</td>
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<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>PHF</td>
<td>Paired helical filaments</td>
</tr>
<tr>
<td>PRI(\alpha)MA</td>
<td>Proline-rich membrane anchor</td>
</tr>
<tr>
<td>SC</td>
<td>Schaffer collaterals</td>
</tr>
<tr>
<td>TLR-4</td>
<td>Toll-like receptor-4</td>
</tr>
<tr>
<td>TNF-(\alpha)</td>
<td>Tumour necrosis factor-(\alpha)</td>
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<td>VMAT</td>
<td>Vesicular monamine transporter</td>
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<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>Acetylcholinesterase</td>
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<td>AD</td>
<td>Alzheimer’s disease</td>
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<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
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<tr>
<td>Ca(^{2+})</td>
<td>Calcium</td>
</tr>
<tr>
<td>ChAT</td>
<td>Choline acetyltransferase</td>
</tr>
<tr>
<td>CHT1</td>
<td>High-affinity choline transporter</td>
</tr>
<tr>
<td>CNO</td>
<td>clozapine-N-Oxide</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DREADD</td>
<td>Designer Receptors Exclusively Activated by Designer Drugs</td>
</tr>
<tr>
<td>FCSRT</td>
<td>Free and Cued Selective Reminding Test</td>
</tr>
<tr>
<td>HDBB</td>
<td>Horizontal diagonal band of Broca</td>
</tr>
<tr>
<td>IGF2</td>
<td>insulin-like growth factor 2</td>
</tr>
<tr>
<td>IL-1(\beta)</td>
<td>Interleukin 1(\beta)</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
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<tr>
<td>LD</td>
<td>Location discrimination</td>
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<td>Abbreviation</td>
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<tr>
<td>LDT</td>
<td>Laterodorsal tegmental nucleus</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTD</td>
<td>Long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>mAChR</td>
<td>Muscarinic acetylcholine receptor</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>mGluR5</td>
<td>Metabotropic glutamate-5 receptor</td>
</tr>
<tr>
<td>MS</td>
<td>Medial septum</td>
</tr>
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<td>MWM</td>
<td>Morris water maze</td>
</tr>
<tr>
<td>nAChR</td>
<td>Nicotinic acetylcholine receptor</td>
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<tr>
<td>NBM</td>
<td>Nucleus basalis of Meynert</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-d-aspartate</td>
</tr>
<tr>
<td>NSC</td>
<td>Neural stem cells</td>
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<tr>
<td>PAL</td>
<td>Paired associates learning</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PPN</td>
<td>Pedunculopontine nucleus</td>
</tr>
<tr>
<td>PrPsc</td>
<td>Cellular prion protein</td>
</tr>
<tr>
<td>PS1</td>
<td>Presenilin 1</td>
</tr>
<tr>
<td>SC</td>
<td>Schaffer collateral</td>
</tr>
<tr>
<td>SGZ</td>
<td>Subgranular zone</td>
</tr>
<tr>
<td>SI</td>
<td>Substantia innominata</td>
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<td>SO</td>
<td>Stratum Oriens</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>STD</td>
<td>Short-term depression</td>
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<tr>
<td>STP</td>
<td>Short-term potentiation</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
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<tr>
<td>TREM2</td>
<td>Triggering receptor expressed on myeloid cells 2</td>
</tr>
<tr>
<td>VACHT</td>
<td>Vesicular acetylcholine transporter</td>
</tr>
<tr>
<td>VDBB</td>
<td>Vertical diagonal band of Broca</td>
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<tr>
<td>VGLUT3</td>
<td>Vesicular glutamate transporter</td>
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Chapter 1

1. Introduction

1.1 Central cholinergic system

Acetylcholine (ACh) was the first neurotransmitter to be identified in the mammalian nervous system (Loewi, 1921), and succeeding decades of research have implicated ACh in regulating vital physiological roles in both the central nervous system (CNS) and peripheral nervous systems (PNS). In the periphery, ACh is a fast-acting neurotransmitter in the autonomic ganglia and the neuromuscular junction (Changeux 2010). Central cholinergic neurotransmission modulates neuronal excitability, coordinates neuronal firing rates, and influences release of other neurotransmitters (Zhang and Sulzer 2004; Higley et al. 2011). Central cholinergic activity has been shown to influence a number of brain functions including attention, memory, sleep, and drug abuse (Reviewed in (Everitt and Robbins 1997; Hasselmo and Giocomo 2006)). Moreover, degeneration of the cholinergic system is thought to underlie some of the
cognitive and behavioural impairments seen in neurodegenerative diseases (Whitehouse et al. 1981; Lehericy et al. 1993).

ACh in the CNS originateS mainly from projection neurons, which innervate target cells in various areas of the brain (Figure 1) (Reviewed in (Picciotto et al. 2012)). Most cholinergic projection neurons originate from nuclei found in the medial habenula (Ren et al. 2011), the basal forebrain nuclei, which consists of the nucleus basalis of Meynert (NBM) (extending to the substantia innominata; SI, in the rodent brain), the medial septum (MS), the horizontal and vertical diagonal bands of Broca (HDBB and VDBB) (Mesulam et al. 1983; Zaborszky 2002), as well as the brainstem cholinergic nuclei, which is comprised of the pedunculopontine nucleus (PPN), and the laterodorsal tegmental nucleus (LDT) (Mesulam et al. 1984) (Figure 1).

Basal forebrain cholinergic nuclei provide vast innervations throughout the mammalian brain. In the early 80’s Mesulam and colleagues assessed the basal forebrain cholinergic system in the rat and classified the nuclei according to their innervated regions (Mesulam 1983). Cholinergic neurons projecting from the MS and VDB to the hippocampus were referred to as Ch1, and Ch2, respectively (Figure 1). The main source of cholinergic neurotransmission to the hippocampus is the MS, which is also referred to as the septohippocampal pathway (Lewis and Shute 1967). The hippocampus receives approximately 90% of its cholinergic input from the MS through the fimbria-fornix fibers, which then innervates the
hippocampus through the stratum oriens (SO) (Dutar et al. 1995). Cholinergic projections from the HDB, referred to as Ch3, project predominantly to the olfactory bulb while NBM and SI projections, referred to as Ch4, innervate the neocortex and the amygdala. Other neuronal types are also part of the basal forebrain nuclei, including GABAergic neurons, glutamatergic neurons, neuropeptides (neuropeptide Y, somatostatin) and neurons containing different calcium binding proteins (calbindin, calretinin, and parvalbumin) (Gritti et al. 2003; Zaborszky et al. 2005). These basal forebrain cholinergic nuclei have been implicated in regulating several aspects of cognition, including memory (Dunnett and Fibiger 1993; Woolf 1997) and attention (Sarter and Bruno 1997).

According to Mesulam's nomenclature (Mesulam et al. 1983), the brainstem cholinergic system is subdivided into Ch5, which refers to cholinergic neurons in the PPN, and Ch6, which refers to cholinergic neurons in the LDT. Both Ch5 and Ch6 cholinergic nuclei predominantly innervate the thalamus (Figure 1). Additionally, PPN cholinergic nuclei innervate the posterior lateral hypothalamus (Ford et al. 1995), the midbrain, the inferior and superior colliculi and pontine regions (Mena-Segovia et al. 2008). These PPN cholinergic neurons have been associated with sleep and wakefulness, and memory (Kessler et al. 1986; Lima et al. 2012).

In the striatum, cholinergic innervation comes mainly from interneurons that are characterized by tonically active ACh-release. Striatal cholinergic interneurons
comprise approximately 1% of striatal cells (Phelps et al. 1985) and have been proposed to play a critical role in behavioural flexibility (Okada et al. 2014).

Figure 1. Schematic overview of the rodent central cholinergic system. Cholinergic striatal interneurons provide local innervation while projection neurons in the basal forebrain system: medial septum (MS;Ch1), vertical limbs of the diagonal band of Broca (VDB;Ch2) and horizontal limbs of the diagonal band of Broca (HDB;Ch3), nucleus basalis of Meynert (NBM;Ch4), and substantia innominate (SI;Ch4) innervate the hippocampus, anterior cingulate gyrus, olfactory bulb, the cortical mantle, respectively. Brainstem cholinergic nuclei: pedunculopontine (PPN;Ch5) and laterodorsal tegmental (LDT;Ch6) innervate the hindbrain, thalamus, hypothalamus, and basal forebrain.
1.1.1 Regulation of central cholinergic signalling

ACh genesis is dependent on the uptake of choline into the presynaptic cell by the high-affinity choline transporter (CHT1). CHT1 acts as the rate-limiting step for ACh production as it regulates the transport of choline from the extracellular space, necessary for ACh synthesis and release, into the presynaptic nerve terminal, (Kuhar and Murrin 1978; Brandon et al. 2004). Choline and intracellular acetyl-CoA are then used for ACh synthesis by choline acetyltransferase (ChAT) (Hersh et al. 1996). ChAT is mainly localized in the cytoplasm of cholinergic nerve terminals, and is regulated by the influx of calcium (Ca$^{2+}$) and phosphorylation by kinases (Rylett 1989; Schmidt and Rylett 1993). ChAT activity is in kinetic excess in cholinergic nerve terminals; hence, ChAT does not appear to represent a rate-limiting step for ACh production (Brandon et al. 2004). Once synthesized, ACh is packaged into synaptic vesicles by the vesicular acetylcholine transporter (VACHT) to form a quantum, which determines the minimum amplitude of a postsynaptic potential (Fatt and Katz 1951, 1952)(Reviewed in (Van der Kloot and Molgo 1994; Prado et al. 2013)). ACh is then released by exocytosis (Figure 2) (Song et al. 1997; Lima Rde et al. 2010).
Once in the synaptic cleft, ACh can bind to muscarinic ACh receptors (mAChRs) or nicotinic ACh receptors (nAChRs). Alternatively, remaining synaptic ACh is degraded by acetylcholinesterase (AChE) (Ellman et al. 1961), a membrane enzyme regulated by a proline-rich membrane anchor (PRiMA) (Perrier et al. 2002) (Figure 2).

A large body of evidence indicates that VAChT activity plays a critical role in regulating ACh storage and release (Reviewed in (Prado et al. 2013)). VAChT is a 12 transmembrane protein with a molecular mass of 75 kDa, that is part of a Major Facilitator Superfamily of transporters (Alfonso et al. 1993; Varoqui and Erickson 1996; Vardy et al. 2004). VAChT is part of the vesicular monamine transporter (VMAT) family (VMAT1 and VMAT2) and belongs to the SLC18 family of proton/neurotransmitter antiporters, which regulate the release of neurotransmitters (Erickson et al. 1994; Reimer et al. 1998). VAChT is found in synaptic vesicles (Varoqui and Erickson 1998; Krantz et al. 2000). To package and transport ACh, VAChT uses the electrochemical gradient generated by a V-type proton ATPase (Parsons et al. 1993; Parsons 2000). VAChT exchanges two luminal protons for a cytoplasmic ACh molecule (Nguyen et al. 1998).

Intriguingly, the VAChT gene lies uninterrupted within the first intron of the ChAT gene and in the same transcriptional orientation (Bejanin et al. 1994; Erickson et al. 1994). Because of this unique organization this genomic site has been called "cholinergic gene locus" (Bejanin et al. 1994; Erickson et al. 1994), a term used
to define a cluster of functionally related genes that are regulated and transcribed as a unit (Jacob and Monod 1961; Eiden 1998). This complex genetic organization allows multiple mRNA expression of VACHT and ChAT in a cell-specific manner throughout the nervous system. Despite this unique organization, evidence suggests that there are independent regulatory processes specific for either VACHT or ChAT as well as shared regulatory elements. For example, it has been demonstrated that VACHT and ChAT genes require specific silencer elements to repress expression of these genes in non-cholinergic cells (De Gois et al. 2000). On the other hand, studies in cell lines suggest independent regulation of ChAT and VACHT mRNA levels (Malik et al. 2000; Dolezal et al. 2001).

A number of studies highlight the importance of VACHT in regulating cholinergic signalling across species. In Caenorhabditis elegans (C. elegans), mutations that eliminate the VACHT gene (named unc-17) function were lethal (Alfonso et al. 1993), highlighting the physiological importance of VACHT in storage of ACh. Likewise, VACHT expression in rodents (Erickson et al. 1994; Prado et al. 2006) and humans (Roghani et al. 1994) is critical for ACh storage and release, as genetic knockout VACHT mice die shortly after birth (de Castro et al. 2009). VACHT expression is essential for regulating the amount of ACh stored and released. For example, overexpression of VACHT in Xenopus neurons significantly increased the number of miniature excitatory postsynaptic potentials (EPSPs), suggestive of an increase in vesicles storing ACh (Song et al. 1997). In
vivo studies showed that decreased VACHT levels severely compromise packaging of ACh into synaptic vesicles and therefore reduce ACh release by nerve terminals (Prado et al. 2006; de Castro et al. 2009; Lima Rde et al. 2010). In mice with 70% reduction in VACHT levels, electrophysiological recordings in neuromuscular junctions demonstrated marked reductions in endplate potentials and miniature endplate potentials, indicative of quantal content, compared to wild-type controls (Lima Rde et al. 2010). Conversely, overexpression of VACHT enhances ACh secretion (Song et al. 1997; Nagy and Aubert 2012; Kolisnyk et al. 2013). Indeed, due to the unique organization of the cholinergic gene locus, transgenic mice generated using a bacterial artificial chromosome (BAC) containing the ChAT gene to express channelrhodopsin-2 protein under the ChAT promoter present elevated mRNA and protein VACHT levels (Kolisnyk et al. 2013). Importantly, these mice presented a functional increase in synaptic ACh levels along with impairments in cognition and enhanced physical endurance (Kolisnyk et al. 2013). These findings indicate that VACHT levels regulate ACh storage and release.
Figure 2. Schematic representation of a cholinergic synapse. Choline is taken up into presynaptic cell by CHT1, once in the nerve terminal ChAT synthesizes ACh and is loaded and released by VACHT. Once in the synapse ACh signals through mAChRs (M), or nAChRs (N). Remaining ACh in the synapse is degraded into acetate and choline by AChE. Key represents unlabeled symbols due to space limitations.
1.1.2 *Muscarinic receptors*

ACh action is mediated through two classes of receptors: muscarinic acetylcholine receptors and nicotinic acetylcholine receptors (mAChRs and nAChRs, respectively). Muscarinic receptors consist of 5 receptor subtypes (M1-M5), which are prototypical members of the superfamily of G-protein-coupled receptors, and are found both pre- and post-synaptically throughout the brain (Wess 1996). mAChR subtypes M1, M3, and M5 are linked to the G_q proteins, which activate phospholipase C, whereas M2 and M4 subtypes are coupled to G_{i/o} type G-proteins, which negatively couple to adenylate cyclase (Wess 1996; Wess et al. 2003).

The five-receptor subtypes of the mAChR are expressed throughout the brain in both neurons and glia, and most cell types express at least two or more subtypes (Wess 1996; Caulfield and Birdsall 1998; Volpicelli and Levey 2004). The M1, M4, and M5 mAChR subtypes are mainly found in the CNS while the M2 and M3 mAChR subtypes are expressed in both the PNS and the CNS (Wess 1996; Wess et al. 2007). ACh-mediated activation of mAChRs has been shown to modulate an array of phospholipases, ion channels, and protein kinases through activated G-protein α-subunits and βγ complexes (Wess 1996; Nathanson 2000; Lanzafame et al. 2003). These diverse biochemical effects lead to distinct electrophysiological responses depending on the subtype of cell stimulated (Wess 1996; Wess *et al.* 2007). The levels of expression of each subtype vary...
within brain regions; for example, in humans, the M1 mAChR subtype is highly expressed (35-60% of all mAChR binding sites) in regions associated with cognitive processes such as the hippocampus and the neocortex (Volpicelli and Levey 2004). In contrast, M2 and M4 mAChR subtypes are abundantly found in the basal forebrain, and the striatum, respectively (Levey et al. 1991). The M3 and M5 mAChR subtypes are expressed at low levels in the CNS (Levey et al. 1991). Diverse effects could occur depending on the location as well as the mAChR subtype stimulated. For instance, post-synaptic stimulation of M1/M5 receptors increases neuronal excitability of cortical neurons (Douglas et al. 2002), while presynaptic M2 receptors decrease glutamate release from corticostriatal synapses (Higley et al. 2009). M2 receptors located on presynaptic cholinergic nerve terminals in the hippocampus and cortex mediate inhibition of ACh release (Quirion et al. 1995; Zhang et al. 2002). Thus, central M2 receptors have been proposed as a potential pharmacological target in that blocking M2 receptor activity would enhance cholinergic signalling in AD patients (Quirion et al. 1995; Carey et al. 2001).

In vivo, activation of M1 mAChRs modulates excitatory synaptic transmission via N-methyl-D-aspartate (NMDA)-receptor potentiation (Marino et al. 1998). M1 knockout mice exhibit selective cognitive impairments in tasks requiring interactions between the hippocampus and cortex (Anagnostaras et al. 2003). Moreover, evidence shows that activation of M1 mAChRs induces long-term potentiation (LTP), suggesting that M1 mAChRs could play a role in regulating
hippocampal plasticity (Buchanan et al. 2010; Giessel and Sabatini 2010; Dennis et al. 2015). Because of the low expression of M3 mAChRs in the CNS, little is known regarding their role in cognition; however, M3 mAChRs have been implicated in regulating smooth muscle contractility and salivary secretion (Caulfield 1993; Eglen et al. 1996). Similar to M1 mAChR knockout mice, M5 mAChR knockout mice have impaired hippocampal LTP, as well as deficits in the Y-maze task and object recognition (Araya et al. 2006). Additionally, M2 knockout mice display impairments in working memory, cognitive flexibility, and hippocampal plasticity (Seeger et al. 2004). Mice lacking the M4 mAChR subunit, however, showed no impairments in spatial and working memory, while social interaction was abnormal in M4 knockout mice compared to controls (Koshimizu et al. 2012). Furthermore, evidence shows that M4 mAChRs play an important role in regulating locomotion, as well as modulating prepulse inhibition (Gomeza et al. 1999; Felder et al. 2001). These findings illustrate the distinct behavioural outcomes by modulating each muscarinic receptor subtype in the CNS.

1.1.3 Nicotinic receptors

Nicotinic receptors are non-selective, excitatory cation channels with a molecular mass of 290 kDa (Changeux et al. 1998; Picciotto 2003). These receptors are encoded by 12 genes, and occur as homomeric or heteromeric clusters of a large family of α- (α2-α10) and β- (β2-β4) subunits (Gotti et al. 2007). Nicotinic receptors are permeable to sodium (Na⁺), potassium (K⁺), and Ca²⁺ ions,
depending on their subtype composition (Gotti et al. 2006). In the CNS, the two main nicotinic receptor subtypes are the homomeric α7, and the heteromeric α4β2 subunits (Hill et al. 1993; Zoli et al. 1995). One of the differences regarding these nAChRs is their propensity to desensitization. α7 nAChRs are rapidly desensitized to high concentrations of ACh while α4β2 nAChRs are slowly desensitized (McGehee and Role 1995; Mansvelder et al. 2002). Experiments demonstrate high levels of expression of α7 and α4β2 in the hippocampus (Seguela et al. 1993); and indicate that these receptors mediate distinct forms of hippocampal plasticity (Ji et al. 2001). nAChRs have high affinity for nicotine and low concentrations of nicotine significantly enhance excitatory transmission in hippocampal neurons (Gray et al. 1996). Activation of nAChRs excites target cells and it is associated with fast synaptic transmission through regulating the release of many neurotransmitters, including ACh (Vidal and Changeux 1993; Marchi and Raiteri 1996). For example, nicotinic stimulation enhances ACh release from murine synaptosomes (Rowell and Winkler 1984), while the use of nicotinic antagonists in striatal brain slices attenuates evoked dopamine release by 90% (Zhou et al. 2001). Experiments show that this modulation of neurotransmitter release is region-, and subtype-specific. For instance, α4/α6β2 nAChRs mediate the release of dopamine in the striatum, while the α3β4 nAChRs mediate ACh release in the intrapeduncular nucleus (Grady et al. 2001). Furthermore, the location of nAChRs dictates the effects they would have on neurotransmitter release and synaptic plasticity (Ji et al. 2001; Gu and Yakel 2011). That is, activation of α7 nAChRs on hippocampal interneurons blocks LTP
responses in pyramidal cells, while presynaptic activation of nAChRs in pyramidal cells increases glutamate release and LTP (Ji et al. 2001). This suggests that nAChRs, through pre and postsynaptic mechanisms, are involved in regulating synaptic plasticity through distinct receptor subtypes.

At the behavioural level, nAChRs have been majorly implicated in mediating cue detection during attentional performance (McGaughy et al. 1999; Parikh et al. 2010). Indeed, there is strong evidence linking nicotine to enhancement of attention in humans (Lawrence et al. 2002), and rodents (Young et al. 2004). Local infusions of nicotine into the prefrontal cortex in rats significantly enhance response accuracy in the five choice serial reaction time task (5-CSRTT) (Hahn et al. 2003). Special focus has been on investigating the effects of β2-containing nAChRs and α7 nAChRs, which are widely expressed in the brain (Gotti et al. 2007). For example, the use of α7 agonist R3487/MEM 3454 and 5-HT3 receptor antagonist improves sustained attention in rats (Rezvani et al. 2009). Furthermore, low dose injections of α7 agonists (PHA-543,613, and PNU-282,987) improves attentional performance of wild-type mice, while high doses impair attentional performance in the 5-CSRTT (Kolisnyk et al. 2015). Interestingly, α7 nAChR knockout mice display impairments in attentional performance in the 5-CSRTT; these deficits however, were reversed by administration of β2 nAChR agonist (Kolisnyk et al. 2015). Indeed, mice lacking the β2 nAChR subunit exhibit impairments in attentional performance in the 5-CSRTT (Guillem et al. 2011). Interestingly, these deficits were reversed by
reexpression of the β2 subunit by viral injections of a β2-eGFP bi-cistronic vector in the prelimbic region of medial prefrontal cortex (Guillem et al. 2011). These findings suggest that α7 and β2 nAChRs play a critical role in regulating attention.

1.2 Alzheimer’s Disease

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder first described by Alois Alzheimer in 1906, and is the most common form of dementia. Dementia refers to a class of disorders characterized by deterioration of thinking ability and memory. Behavioural and psychological symptoms of dementia include memory loss, reasoning loss, attention deficits, delusions, hallucinations, agitation, and depression (Finkel et al. 1996; Pinto et al. 2011). Pathological hallmarks of AD and decline in quality of life occur gradually, with dementia being the end stage. Patients diagnosed with AD typically endure a decade of progressive increase in AD-related pathological processes before the presentation of clinical symptoms (Jack et al. 2009). This slow but inevitable progression of AD places a substantial burden on patients, families, caregivers, and society. In 2010, approximately 36 million individuals worldwide were diagnosed with AD, and it was estimated to cost the world an alarming $604 billion (World Alzheimer Report, 2010). These figures are projected to triple by 2050 (World Alzheimer Report, 2010), calling out the need for novel therapeutic targets in AD.
AD is associated with vigorous forebrain neuropathology, with two major pathological hallmarks being amyloid beta (A\beta) plaques and neurofibrillary tangles (Ballard et al. 2011). Both A\beta and neurofibrillary tangles induce progressive neuronal dysfunction and subsequent neuronal death, resulting in severe brain atrophy and loss of cognitive function (Selkoe 2003). Moreover, AD patients also exhibit central vascular impairments, characterized by decreased blood flow to the brain leading to compromised clearance of A\beta (Reviewed in (Zlokovic 2011)). This consequently aids the progressive neuropathology and cell death observed in AD. In addition to these neuropathology processes, loss of cholinergic tone is considered to be one of the earliest pathological events that occur in AD (Perry et al. 1978; Bowen and Davison 1980).

1.2.1 *Amyloid Pathology*

In patients with AD, the formation of amyloid plaques is a key feature that has been proposed to underlie neurodegeneration and deteriorating cognition (Reviewed in (Selkoe and Schenk 2003)). In AD, plaque deposition occurs initially in the basal areas of the neocortex then propagates to the hippocampus, and ultimately propagates to other subcortical regions (Braak and Braak 1991). Amyloidosis refers to the pathological process where deposits of 5-10 nm of amyloid fibrils progressively accumulate in the extracellular spaces of tissues (Selkoe 1991). Amyloid plaques consist mainly of A\beta peptides, which are derived
from the amyloid precursor protein (APP) (Selkoe 2003). APP is a type 1 transmembrane protein that consists of 695-770 amino acids and undergoes endoproteolytic cleaving through one of two pathways: a non-amyloidogenic, and an amyloidogenic (Aβ-yielding) pathway (Reviewed in (LaFerla et al. 2007)). In the non-amyloidogenic pathway, APP is cleaved at the Aβ domain by the α-secretase enzyme, and as a result, a larger ectodomain named sAPPα and a smaller carboxyl-terminal fragment (C83) are generated (Kojro and Fahrenholz 2005). In the amyloidogenic pathway, the β-secretase enzyme cleaves APP, generating the sAPPβ ectodomain and retaining the C99 amino acids peptide membrane-bound. C99 is then cleaved from the amino terminus by the gamma-secretase enzyme to release Aβ. This process yields Aβ1-40 or Aβ1-42 depending on the cleavage site. To note, the 42-residue variant is more hydrophobic than the 40-residue variant of Aβ and is the major constituent of amyloid plaques seen in AD (Jarrett et al. 1993). Mutations in the genes that encode APP or the enzyme presenilin 1 (PS1), which forms the catalytic domain of the larger gamma secretase complex, can lead to AD (De Strooper et al. 1999)(Reviewed in (De Strooper 2010)).

The amyloid hypothesis states that mutations in these genes significantly increase Aβ-42 production and accumulation, which in turn triggers the oligomerization of Aβ-42 leading to formation of amyloid plaques (Hardy and Selkoe 2002). The deposition of Aβ-42 plaques has consequences on synaptic
function and triggers inflammatory responses (Rogers et al. 1996). Furthermore, deposition of Aβ-42 plaques has been thought to contribute to tangle formation due to alterations in kinase activity (Lewis et al. 2001) (Reviewed in (Hardy et al. 1998)).

Several lines of work have demonstrated that soluble oligomers of Aβ are major contributors to the synaptic impairments seen in AD (Lambert et al. 1998; Mucke et al. 2000; Walsh et al. 2002; Ferreira and Klein 2011). Aβ toxicity can be mediated via binding to a wide range of receptors, which activates distinct signalling pathways. For example, the cellular prion protein (PrPc) is an Aβ oligomers-receptor, and has been shown to mediate Aβ oligomers-induced synaptic dysfunction (Lauren et al. 2009). Aβ oligomers have also been shown to bind with high affinity to the α7 nAChR, and have been associated with triggering synaptic dysfunction and cognitive impairments (Wang et al. 2000; Dziewczapolski et al. 2009). Moreover, extracellular Aβ oligomers bind to the cell surface to N-methyl-D-aspartate receptors (NMDARs) and elicit synaptic dysfunction through an increase in production of reactive oxygen species (Snyder et al. 2005; De Felice et al. 2007). In addition, Aβ oligomer-mediated inhibition of NMDAR synaptic plasticity was shown to be mediated by the metabotropic glutamate-5 receptor (mGluR5) (Hu et al. 2014). Studies in hippocampal slices demonstrated that Aβ oligomers lead to an NMDAR-independent long-term depression (LTD), an effect that was blocked by mGluR5.
antagonists (Li et al. 2009). Further experiments have shown that Aβ oligomers and the PrPc form a complex with mGluR5 to induce synaptic toxicity (Um et al. 2013). Additionally, Aβ oligomers were shown to induce nerve growth factor (NGF)-mediated cell death via the induction of the p75 neurotrophin receptor (p75NTR) (Yamamoto et al. 2007), which is part of the tumor necrosis receptor superfamily (Hansma et al. 1995). These pathological mechanisms are thought to be major contributors to the neuronal dysfunction and neuronal cell death observed in dementia (Hardy and Selkoe 2002).

Different mouse models of AD have been generated and widely used to investigate molecular mechanisms leading to pathology and altered cognition. In vivo, imaging studies in mice overexpressing APP and PS1 (APP/PS1) reveal plaque formation at 3 months of age, which in turn leads to oxidative stress and cell death in surrounding cells (Xie et al. 2013). One of the widely used AD mouse models is the 3xTgAD mouse model. This mouse model expresses the human APP Swedish and tau P301L transgenes, and the PS1 M146V knockin (Oddo et al. 2003). The 3xTgAD model develops AD-like pathology in an age-dependent manner, highlighted by plaque deposition, and tangle formation (Oddo et al. 2003; Billings et al. 2005). Studies in the 3xTg-AD mouse model have demonstrated that intracellular Aβ accumulation worsens synaptic health and underlies cognitive impairments (Billings et al. 2005). Moreover, LTP deficits were observed in 3xTg-AD mice prior to plaque and tangle pathology, suggesting that increased levels of intracellular Aβ levels impair synaptic plasticity (Oddo et
Similarly, intracellular $A\beta$ is present in AD patients even prior to plaque and tangle formation, suggesting that intracellular $A\beta$ accumulation is an early event in neuronal pathology (Gouras et al. 2000; Wegiel et al. 2007). However, this is controversial as these results may be due to non-specific binding of the antibody to APP. Nonetheless, extracellular amyloid plaque deposition was found to be poorly linked to the cognitive decline seen in AD (Kelley and Petersen 2007). Moreover, experimental AD models showed that the disruption of connectivity within the hippocampal neural circuitry and synaptic loss caused by $A\beta$ occurred well before plaque formation (Redwine et al. 2003; Wu et al. 2004). In addition to the $A\beta$ toxicity on synaptic integrity, studies have demonstrated that PS1 mutation in the 3xTg-AD mouse model impairs cholinergic-mediated hippocampal plasticity (Wang et al. 2009). Moreover, 6 month-old 3xTg-AD mice display decreased levels of $\alpha 7$ nAChRs in regions where intraneuronal $A\beta$ accumulates, including the hippocampus (Oddo et al. 2005). Interestingly, administration of an M1 agonist in 3xTg-AD mice was shown to be sufficient to rescue cognitive deficits in the spatial Morris Water Maze (MWM), and attenuated $A\beta$ and tau pathology (Caccamo et al. 2006). Additionally, in aged 3xTg-AD mice, treatment with a $\alpha 7$ nAChR agonist significantly reversed cognitive impairments in these mice compared to age-matched controls, however, neuropathological hallmarks including $A\beta$ deposits were unchanged (Medeiros et al. 2014). These findings are in line with a number of studies that have demonstrated that $A\beta$ binds to the $\alpha 7$ nAChR with high
affinity, resulting in receptor internalization and increased intracellular Aβ levels (Wang et al. 2000; Nagele et al. 2002). Indeed, human studies reveal high immunoreactivity of amyloid β in basal forebrain cholinergic neurons in early adult life (Baker-Nigh et al. 2015). These findings suggest that oligomerization of Aβ in these basal forebrain cholinergic neurons during aging could contribute to the neurodegeneration observed in cholinergic neurons in AD.

1.2.2 Tauopathy

Another major hallmark of AD is the formation of neurofibrillary tangles (NFT). Neurofibrillary tangles are mainly composed of hyper-phosphorylated and aggregated forms of tau, a microtubule-associated protein (MAP), which accumulates in neurons leading to neuronal apoptosis (Iqbal and Grundke-Iqbal 2006). The tau protein is encoded my a single MAPT gene on chromosome 17, and is alternatively spliced to six protein isoforms in the adult human brain (Goedert et al. 1989). As a microtubule protein, tau interacts with tubulin to stabilize its assembly into microtubules and aids their structural stability (Weingarten et al. 1975). Tau is found predominantly in neurons and regulates axonal transport, elongation, and maturation (Stamer et al. 2002)(Reviewed in (Wang and Mandelkow 2016)). The biological functioning of tau is regulated by its phosphorylation.
In normal conditions, tau carries approximately two phosphates per molecule, while in AD; phosphorylation of tau is increased to eight phosphates per molecule (Kopke et al. 1993). There are 85 identified potential phosphorylation sites in the tau isoform (24NR) (80 Ser or Thr, and 5 Ty), of which 17 Thr-Pro or Ser-Pro motifs have been linked to the hyperphosphorylation phenotype observed in AD (Hanger et al. 2009). In AD, hyperphosphorylation of tau consequently leads to the accumulation of intracellular neurofibrillary tangles (NFT) and paired helical filaments (PHF) (Kidd 1963). Under pathological conditions, a misbalance of tau binding to microtubules leads to high levels of unbounded tau in the cytosol, which in turn leads to conformational changes that causes aggregation of tau (Reviewed in (Ballatore et al. 2007)). Once aggregated in the cell, pretangles are formed, which ultimately undergo a structural rearrangement to form β-sheet-positive PHFs and NFTs (Galvan et al. 2001; Maeda et al. 2007). Formation of these PHFs and NFTs has been proposed to be dependent on the rate of phosphorylation, which would determine the concentration of unbound tau fractions in the cytosol (Reviewed in (Ballatore et al. 2007)). Furthermore, both total and phosphorylated levels of tau have been effectively correlated to cognitive deficits in AD (Buerger et al. 2002; Kelley and Petersen 2007; Diniz et al. 2008). It has been suggested that modulation of tauopathy in AD occurs via key kinases and phosphatases, including GSK3β and CDK5 (Hanger et al. 1992; Shelton and Johnson 2004). Indeed, transgenic mouse models overexpressing GSK3β showed increased phosphorylation of tau and hippocampal neurodegeneration (Engel et al. 2006). Similarly, transgenic
mice overexpressing CDK5 displayed increased numbers of NFTs (Noble et al. 2003). This suggests that these kinases are key players in triggering AD-tauopathy.

3xTg-AD mice, which express the P301L mutation in tau and APP/PS1 mutations (Oddo et al. 2003), progressively develop tau pathology at 6-12 months of age (Oddo et al. 2003). This manifestation of tau pathology could be identified using antibodies that detect the amount of phosphorylation at specific residues including Ser202, Thr 205, Ser 231, Ser396 and Ser404 (LaFerla and Oddo 2005). 3xTg-AD mice display working memory impairments in the 8-arm radial maze task as early as 2 months (Stevens and Brown 2015). Thus, both Aβ and neurofibrillary tangles seem to induce progressive neuronal dysfunction and subsequent death, resulting in severe brain atrophy and loss of cognitive function (Selkoe 2003).

1.2.3 Neuroinflammation

Neuroinflammation is a secondary response to insult in the CNS that leads to activation of glial cells. Microglia are found throughout the CNS with the highest density of expression in the hippocampus, basal ganglia, and the substantia nigra. In AD, increased microglial activation is a key hallmark, which in turn leads to enhanced release of pro-inflammatory cytokines and abnormal phagocytosis. The pro-inflammatory cytokines released by microglia include tumour necrosis
factor-α (TNF-α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and reactive oxygen species (Reviewed in (Block et al. 2007)). In AD, over activation of microglia precedes the development of cognitive impairments and are typically found surrounding aggregated Aβ plaques (McGeer et al. 1987; Veerhuis et al. 1999). In early phases of AD, these microglia are thought to contribute to Aβ clearance (El Khoury et al. 2007). In late AD however, evidence shows that Aβ plaques activate microglia, which releases neurotoxic, and proinflammatory factors that ultimately trigger neuronal death (Hickman et al. 2008). Recent work has demonstrated that mutations in the Triggering receptor expressed on myeloid cells 2 (TREM2) is involved in increasing the risk of developing AD (Jonsson et al. 2013). TREM2 is expressed by microglia and experiments show that loss of one copy of TREM2 significantly increases Aβ plaque accumulation in 5xFAD mice (Wang et al. 2015). Furthermore, recent findings illustrate that administration of interleukin 33 (IL-33) rescues synaptic plasticity deficits in an APP/PS1 AD mouse model (Fu et al. 2016). Post administration of IL-33 injections, soluble Aβ levels were significantly reduced by increased microglial phagocytic activity (Fu et al. 2016). These findings suggest that the innate anti-inflammatory response may serve as a target for AD.

In mouse models of AD, the inflammatory response is less severe and does not precede the hallmarks of the disease as seen in humans (Reviewed in (Duyckaerts et al. 2008)). Nonetheless, 3xTgAD mice presented early microglial activation (3 months of age), which preceded plaque Aβ deposition (12 months of
age) (Janelins et al. 2005). Interestingly, this inflammatory response was characterized by increased expression of the pro-inflammatory markers: TNF-α and monocyte chemoattractant protein-1 (MCP-1), which coincided with the high accumulation of intracellular Aβ at 3 months of age (Janelins et al. 2005).

A number of studies trigger neuroinflammation responses using injections of lipopolysaccharide (LPS) (Hauss-Wegrzyniak et al. 1998; Kitazawa et al. 2005), which is an endotoxin that induces a cellular stress response. Upon administration, LPS forms a complex with CD14, which interacts with the toll-like receptor-4 (TLR-4), and activates microglia leading to secretion of pro-inflammatory cytokines including IL-1, IL-6, IL-12, and TNF-α (Reviewed in (Rivest 2009)). Indeed, experiments involving neuroinflammation in rats using ventricular infusions of LPS to the basal forebrain have demonstrated increased microglial activation in the basal forebrain (Wenk et al. 2000). Chronic basal forebrain LPS infusion significantly decreases activity of ChAT in the basal forebrain (Willard et al. 1999) and triggers activation of caspase-3, caspase-8, and caspase-9 activity in the ventral caudate region (Wenk et al. 2000). These findings suggest that cholinergic neurons are selectively vulnerable to the toxic effects of chronic neuroinflammation. In line with these findings, data from separate studies demonstrate that systemic administration of cholinesterase inhibitor, galantamine, significantly suppresses TNF-α expression induced by systemic LPS injections (Pavlov et al. 2009). Interestingly, this effect was blocked by administration of atropine, a muscarinic receptor antagonist, suggesting a
critical role for cholinergic signalling in central inflammatory responses (Pavlov et al. 2009).

Studies using infusion of LPS in the fourth ventricle show that 2 weeks after last of infusion of LPS, microglial activation was substantially attenuated in all areas of the brain except the hippocampus, suggesting a regional vulnerability to neuroinflammation (Hauss-Wegrzyniak et al. 1998). The findings of these studies indicate that while systemic-induced inflammation provides insight regarding the inflammatory cellular responses in the CNS, it is not an ideal approach to model the chronic inflammatory responses observed in AD. This is due to the confounding factor of systemic inflammation induced by using LPS. Moreover, using mouse models overexpressing specific cytokines may also not provide a faithful model of the endogenous inflammatory responses observed in AD.

The recent development of Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) has provided means of activating or inhibiting a specific cell type (Armbruster et al. 2007)(Reviewed in (Roth 2016)). This technology utilizes engineered-GPCRs that bind to designed ligands to elicit either silencing or enhancement of neuronal or non-neuronal activation. One of these engineered receptors is the human Gαq M3-muscarinic receptor (Schmidt et al. 2003; Armbruster et al. 2007). The modified receptor, referred to as hM3Dq, does not bind ACh but can be activated by the inert metabolite clozapine-N-Oxide (CNO), to trigger Gα-q-mediated signalling pathways.
Modified M4-muscarinic receptors are also available. Armbruster et al. demonstrated that the use of CNO on hM4Di-DREADD in hippocampal neurons elicits neuronal inhibition through Gαi-mediated signalling (Armbruster et al. 2007). Indeed, increasing evidences show in vivo CNO mediated activation or silencing of neurons using either hM3Dq-DREADD or hM4Di-DREADD, respectively (Krashes et al. 2011; Ray et al. 2011; Atasoy et al. 2012; Garner et al. 2012).

A recent study demonstrated that activation of M3 mAChRs modulates microglia function (Pannell et al. 2016). Using calcium imaging in cultured adult mouse cortical microglia, it was determined that 8% of cultured microglia responded to carbachol. In the same study, treatment of the adult cortical microglia with interferon-gamma, a pro-inflammatory cytokine, resulted in an increase in the number of carbachol-sensitive microglia to 60%. Moreover, analysis of the expression of the M3 mAChR using quantitative PCR (qPCR) showed an upregulation in adult cortical microglia treated with interferon-gamma, an effect that was blocked by treatment with an M3-specific antagonist (Pannell et al. 2016). Importantly, these elegant experiments demonstrated that microglia that had upregulation of M3 mAChR also expressed the major histocompatibility complex (MHC-I and MHC-II), indicative of functionally activated microglia (Pannell et al. 2016). Due to the lack of a faithful model of central inflammatory response seen in AD, DREADDs may serve as a tool to investigate how chronic
activation of central inflammatory responses plays a role in the pathogenesis of AD.

1.2.4 *Cholinergic dysfunction*

Bartus and colleagues were the first to formulate the cholinergic hypothesis in AD (Bartus et al. 1982). This hypothesis states that loss of central cholinergic neurotransmission plays a key role in the cognitive decline observed in AD. Moreover, deterioration of basal forebrain cholinergic neurons has been positively correlated with cognitive impairments in AD (Perry et al. 1978; Collerton 1986; DeKosky et al. 1992). This is mainly highlighted by loss of different cholinergic markers such as ChAT (DeKosky et al. 1992), AChE (DeKosky et al. 1992), and VAChT (Chen et al. 2011). As such, decades of research led to the development of FDA approved cholinergic drugs to treat AD: donepezil, galantamine, and rivastigmine (Schneider et al. 2014). Cholinergic agonists approved for treatment in AD enhance central cholinergic signalling by inhibiting the activity of the acetylcholinesterase enzyme, which normally degrades synaptic ACh (Massoulie et al. 1993)(Reviewed in (Buccafusco and Terry 2000). However, evidence shows that these cholinergic agents present modest effects in treating patients with mild cognitive impairment (MCI) (Doody et al. 2009), an early cognitive impairment proposed to precede AD. The modest effect of these clinical trials could be due to heterogeneity of the MCI-diagnosed patients as many of the symptoms of MCI may be caused by different disorders,
and that not all MCI patients necessarily develop AD (Petersen et al. 1999; Schneider et al. 2014). The development of the Free and Cued Selective Reminding Test (FCSRT) was shown to detect prodromal AD within MCI patients (Sarazin et al. 2007). Interestingly, in prodromal AD patients, an annual daily dose of donepezil (10mg) reduced the annual percentage change of total hippocampal volume by 45% (Dubois et al. 2015). This suggests that donepezil may play a protective role on hippocampal atrophy and cognition in patients at-risk of developing AD.

Imaging studies demonstrate progressive neurodegeneration in the basal forebrain cholinergic system, the hippocampus, and cortex in AD, and in patients with MCI (Teipel et al. 2005; Grothe et al. 2010; Grothe et al. 2012; Kilimann et al. 2016). Indeed, magnetic resonance imaging (MRI) experiments reveal that decreased volume of the basal forebrain and the hippocampus significantly predicts conversion from MCI to AD (Brueggen et al. 2015). Moreover, postmortem analysis of protein and mRNA levels of VACHT demonstrated substantial decrease in the levels of VACHT in the frontal cortex of AD patients, indicating that storage and release of ACh is compromised in AD (Chen et al. 2011). Similarly, there is a reduction in expression of M2 mAChR subtype and nicotinic receptors in cortical areas in patients with AD (Mash et al. 1985; London et al. 1989). Despite the substantial cholinergic depletion in projection regions of the basal forebrain cholinergic system, striatal interneurons and thalamic cholinergic signalling from the brainstem cholinergic nuclei remain preserved in
AD (Mesulam 2004). This highlights the selective vulnerability of the basal forebrain cholinergic system and its projections sites, including the cortex and hippocampus in AD.

Mounting evidence has linked the long-term use of drugs with anticholinergic properties to cognitive decline and increased dementia (Ancelin et al. 2006; Carriere et al. 2009; Jessen et al. 2010; Fox et al. 2011; Gray et al. 2015). Indeed, use of the anticholinergic drug oxybutynin chloride for more than 3 years has been shown to increase risk of developing dementia (Gray et al. 2015). Use of drugs with anti-muscarinic activity for more than 2 years in Parkinson’s disease (PD) patients triggered approximately a 2-fold increase in amyloid deposition, compared to untreated controls (Perry et al. 2003). Similarly, there was a significant increase in neurofibrillary tangle densities in patients treated with these anti-cholinergic drugs, compared to untreated controls (Perry et al. 2003). Interestingly, PD patients treated with anti-muscarinic drugs for less than 2-years did not increase levels of amyloidosis nor tangle formation (Perry et al. 2003). This suggests that long-term loss of cholinergic signalling may be related to AD-like pathology.

1.3 Cholinergic regulation of hippocampal function

A large number of studies demonstrate that cholinergic neurotransmission plays a critical role in modulating hippocampal LTP, a molecular correlate of learning
and memory. In *vitro*, experiments show that cholinergic agonists facilitate LTP (Auerbach and Segal 1996). Likewise, in *vivo* stimulation of the septohippocampal pathway enhanced LTP (Galey et al. 1994). Conversely, lesions to the septohippocampal pathway and administration of scopolamine, a mAChR antagonist, attenuated hippocampal LTP, suggesting that cholinergic signalling via mAChR activity could mediate hippocampal synaptic plasticity (Leung et al. 2003). Indeed, M2 mAChR-deficient mice had abolished hippocampal short-term potentiation (STP) and a significant reduction of LTP after high-frequency stimulation (Seeger et al. 2004). Moreover, there is evidence suggesting that M1 mAChRs could play a role in regulating hippocampal plasticity (Wess 2004; Buchanan et al. 2010; Dennis et al. 2016).

The involvement of nicotinic receptor activity has also been heavily implicated in mediating forms of hippocampal synaptic plasticity. In *vitro* application of nicotine on hippocampal brain slices facilitates LTP (Fujii et al. 1999). Moreover, experimental evidence using optogenetics to activate cholinergic inputs reveals that induction of distinct forms of hippocampal synaptic plasticity is timing-dependent (Gu and Yakel 2011). Optogenetic activation of cholinergic input to the CA1 100 milliseconds (ms) prior to Schaffer collaterals (SC) stimulation resulted in α7 nAChR-mediated LTP, while 10 ms prior to SC stimulation led to short-term depression (STD) (Gu and Yakel 2011). Additionally, activation of cholinergic signalling 10 ms after SC stimulation induced mAChR-mediated LTP
(Gu and Yakel 2011). These results demonstrate the temporal precision of the septohippocampal pathway in modulating distinct forms of synaptic plasticity.

Data from in vivo microdialysis studies in rats demonstrate that hippocampal ACh levels are increased in learning and memory tasks (Hironaka et al. 2001; Chang and Gold 2003). Moreover, systemic administration of cholinergic agonists reverses memory impairments induced by lesions to the medial septum or fornix in rats (Decker et al. 1992; Levin et al. 1993). Notably, hippocampal infusions of physostigmine, an AChE inhibitor, rescues memory impairments induced by intra-septal inactivation by muscimol (GABA agonist) infusions in rats (Degroot and Parent 2000). This suggests a critical role of hippocampal cholinergic signalling in modulating memory-related behaviours.

Selective elimination of cholinergic neurons from medial septum using immunotoxins in rodents has also been used to investigate the role of ACh signalling in the hippocampus and cortex. It is somewhat controversial whether selective 192 IgG-saporin lesions of septohippocampal cholinergic neurons can lead to significant impairments in hippocampal-dependent learning and memory tasks in rodents. This is because some reports find a modest effect of lesions on memory tasks (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 myriad of deficits (Walsh et al. 1996; Janis et al. 1998; Gil-Bea et al. 2011). The inconsistency in these studies could be attributed, at least in part, to cholinergic...
neurons expressing more than one class of neurotransmitter transporters and secreting two 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; Munster-Wandowski et al. 2016). Therefore, it is difficult to interpret results with toxin lesions for specific contributions of neurotransmitters in neurons that release 2 chemical messengers. Importantly, evidence suggests that GABA can be released from basal forebrain cholinergic projection neurons. Optogenetic activation of cortical cholinergic axons induce GABA-mediated inhibitory postsynaptic currents in cortical neurons (Saunders et al. 2015). Conversely, selective deletion of the GABA transporter in cholinergic neurons abolished the postsynaptic responses mediated by optogenetic cholinergic activation (Saunders et al. 2015).

In the striatum, glutamate is co-released with ACh due to the presence of the vesicular glutamate transporter (VGLUT3), which is highly expressed in striatal cholinergic interneurons (Guzman et al. 2011; Higley et al. 2011). Cotransmission by cholinergic neurons needs to be considered when investigating the role of ACh in behaviour studies using neuronal lesions. Here we use genetic targeting of either VACHT or ChAT using the Cre/lox system to interfere with ACh release, which has provided a way for investigating specific contributions of ACh when there is co-transmission (Guzman et al. 2011; Martyn et al. 2012; Kolisnyk et al. 2013; Prado et al. 2013).
1.3.1 *Behavioural tasks to assess hippocampal function*

Since it was developed by Richard Morris (Morris 1984), the Morris water maze (MWM) has been widely utilized by researchers to assess spatial learning and memory in animals. The spatial version of this task involves an open circular pool that is filled with water, with a small platform submerged in a fixed location. An animal is placed in the pool from each of four different starting locations, and is required to use distal cues placed around the pool to search for the hidden platform (See Chapter 2.13). Performance in the MWM task depends on intact hippocampal function (Morris et al. 1982; Brandeis et al. 1989)(Reviewed in (D'Hooge and De Deyn 2001)). For example, septohippocampal lesion studies in rats cause impairments in spatial acquisition of the MWM (Morris et al. 1982). Moreover, evidence suggests that performance in this task is dependent on NMDAR function (Morris et al. 1986; Bannerman et al. 1995), and correlates with hippocampal long-term potentiation (Jeffery and Morris 1993; Moser et al. 1998).

While the MWM provides insight regarding spatial navigation in rodents, evidence shows that performance in the MWM can be affected by stress (Holscher 1999). Hence, considering that performance in the MWM is aversively motivated, one cannot exclude motivation and the level of stress as potential factors in the animals’ performance in this task.
The recent development of automated touchscreen behavioural testing for rodents has improved cognitive testing. These touchscreen tasks are analogous to the paradigms and methodologies used in humans, hence, facilitating translational studies between rodents and humans (Morton et al. 2006; Talpos et al. 2009; Talpos et al. 2010; McCarthy et al. 2011; Romberg et al. 2011). One of the touchscreen tasks available is Paired Associates Learning (PAL). This task requires subjects/animals to associate objects with their matched locations on an automated touch screen. Performance in PAL, as well as in other paired-associate tasks, depends on intact hippocampal function in humans and rats (Talpos et al. 2009; de Rover et al. 2011) Deficits in the PAL test have been detected consistently in AD (Blackwell et al. 2004) and schizophrenia (Wood et al. 2002; Barnett et al. 2005). PAL performance has been shown to correlate with severity of affective symptoms and daily functioning in schizophrenia (Barnett et al. 2005). In dementia, PAL has been shown to differentiate between MCI and AD (Blackwell et al. 2004).

Another cognitive domain that is associated with intact hippocampal function is pattern separation. Pattern separation is defined as the filtering of similar representations into less similar outputs (Colgin et al. 2008) and is one of the functions associated with newly born neurons in the dentate gyrus region of the hippocampus (Treves et al. 2008). Specifically, the two-choice location discrimination task has been widely used as a functional assessment of adult hippocampal neurogenesis. This task requires rodents to discriminate between
spatially proximate stimuli (Clelland et al. 2009; McTighe et al. 2009). Lesions in
the dentate gyrus impaired pattern separation in rats using a different task
(Gilbert et al. 2001), while low dose of X-irradiation to ablate hippocampal
neurogenesis in mice disrupted pattern separation in the two-choice location
discrimination task (Clelland et al. 2009). Conversely, genetic deletion of the pro-
apoptotic BAX gene in Nestin-positive NSCs significantly increased newborn
survival cells in the hippocampus and neurogenesis-mediated LTP (Sahay et al.
2011). Importantly, genetic deletion of BAX in Nestin expressing NSCs enhanced
pattern separation (Sahay et al. 2011), supporting an important role of
hippocampal NSCs in modulating pattern separation. Moreover, fMRI studies in
humans reveal specific activity of the dentate gyrus during pattern separation
(Bakker et al. 2008). Together, these results suggest that the dentate gyrus
region of the hippocampus plays a critical role in filtering spatially related
contexts.

1.3.2 Regulation of adult hippocampal neurogenesis

Neurogenesis is defined as the production of new neurons, and occurs mainly in
two regions of the adult mammalian brain; the subgranular zone (SGZ) of the
dentate gyrus in the hippocampus, and the subventricular zone (SVZ) of the
lateral ventricle (Altman 1969; Cameron and McKay 1998; Eriksson et al. 1998).
These regions are referred to as neurogenic niches due to the unique complex
microenvironment critical for neural stem cell (NSC) development (Ma et al.
In the adult hippocampus, new neurons are generated in the SGZ then differentiate into mature neurons in the granular cell layer of the dentate gyrus, where they are functionally integrated into the hippocampal circuitry (van Praag et al. 2002). There are two types of NSCs in the SGZ: type 1 cells refer to a radial dormant population of GFAP, SOX2, and Nestin-positive cells (Figure 3) (Mu et al. 2010); and type 2 cells are composed of actively non-radial proliferating cells (Sox2-positive cells) that generate both astrocytes and neuroblasts (Doublecortin-positive, Figure 3) (Mu et al. 2010). Not all neurogenic cells survive; only approximately 50% of newly born cells become functionally integrated into the hippocampal circuitry in the rat (Cameron and McKay 2001).

The surviving cells in the SGZ project their dendrites to the molecular layer of the dentate gyrus and send projections to CA3 pyramidal cells, becoming fully mature neurons (NeuN-positive) that are integrated into the hippocampal circuitry (Figure 3). A large number of signalling molecules are involved in regulating neurogenesis, including the Wnt signalling pathway. Activation of the Wnt/β-catenin pathway enhances NSC proliferation expression (Kuwabara et al. 2009). Wnt signalling through the GSK3β/β-catenin pathway maintains the potency of neural progenitor cells (Wexler et al. 2009). Indeed, selective inhibition of Wnt signalling in the dentate gyrus of the hippocampus significantly decreases the number of newly born neurons (Lie et al. 2005; Clelland et al. 2009; Jessberger et al. 2009) suggesting a critical role of Wnt signalling in adult hippocampal neurogenesis.
On the other hand, newly born neurons in the SVZ migrate anteriorly to the olfactory bulb through the rostral migratory system, where they become functional local interneurons (Altman 1969; Kornack and Rakic 2001). Adult SVZ neurogenesis has been thought to be associated with subependymal astrocytes, which function as NSCs to produce Mash1-positive progenitors (Parras et al. 2004; Paez-Gonzalez et al. 2014). These cells then differentiate into Doublecortin-positive neuroblasts before migrating to the olfactory bulb. Experiments have shown that adult SVZ neurogenesis is mainly involved in mating (Mak et al. 2007), and in adult paternal recognition of offspring (Mak and Weiss 2010).

Regulation of adult neurogenesis in the SVZ has been demonstrated to be associated with a number of neurotransmitters, including ACh (Liu et al. 2005; Platel et al. 2010)(Reviewed in (Young et al. 2011)). In vitro studies show that ACh significantly increases Doublecortin-positive cells (Paez-Gonzalez et al. 2014). Moreover, Peaz-Gonzalez et al. identified a population of cholinergic neurons that innervate the SVZ neurogenic region. Using optogenetics the authors demonstrated that these cholinergic neurons modulate SVZ neurogenic proliferation via fibroblast growth factor receptor signalling (Paez-Gonzalez et al. 2014). These elegant experiments highlight the importance of cholinergic signalling in regulating adult neurogenesis in the SVZ (Paez-Gonzalez et al. 2014).
The functionality of these cholinergic neurons in adult SVZ neurogenesis-mediated behaviours however, remains undetermined.

Newly born neurons in the hippocampus play an essential role in learning and memory (Gould et al. 1999; van Praag et al. 1999). An increase in neurogenesis by exercise (van Praag et al. 1999) is associated with enhanced hippocampal synaptic plasticity and improved cognitive performance in mice (van Praag et al. 1999). Selective lesions of basal forebrain cholinergic neurons using the immunotoxin 192 IgG-saporin has been shown to significantly decrease neurogenesis in both SVZ and SGZ (Cooper-Kuhn et al. 2004). Moreover, lesions to the basal forebrain cholinergic neurons increased the numbers of apoptotic cells in the subgranular zone of the hippocampus, suggesting that cholinergic input to the hippocampus is required for maintenance of adult neurogenesis (Cooper-Kuhn et al. 2004). Similarly, mice lacking the β2 subunit of the nicotinic acetylcholine receptor display impairments in adult hippocampal neurogenesis, characterized by a decrease in cell proliferation in the dentate gyrus (Harrist et al. 2004). Systemic injections of donepezil, a cholinesterase inhibitor, enhanced survival of newborn neurons, but not proliferation, in both neurogenic regions in mice (Kaneko et al. 2006). Experiments also demonstrate that scopolamine, a muscarinic receptor antagonist, reduced the number of newborn cells in the rat hippocampus. Furthermore, administration of oxotremorine, a non-selective muscarinic agonist, significantly increased cell proliferation in a dose-dependent manner in rats (Van Kampen and Eckman
These findings point to cholinergic signalling as a potential modulator of adult hippocampal neurogenesis.

**Figure 3. Illustration depicting neurogenesis in the subgranular zone in the hippocampus (SGZ).** In the SGZ layer, type 1 cells are a radial dormant population of cells that express GFAP, SOX2, and Nestin. Type 2 NSCs are composed of actively non-radial proliferating cells (Sox2-positive cells) that generate both astrocytes and neuroblasts (Doublecortin-positive) that ultimately become mature neurons (NeuN-positive) and functionally incorporate into the hippocampal circuitry.
1.4 Rationale and Hypothesis

Loss of basal forebrain cholinergic signalling and hippocampal atrophy are major hallmarks in AD (Perry et al. 1978; Bowen and Davison 1980). However, the specific contribution of cholinergic neurotransmission in AD pathology and cognitive deficits still remains unclear.

The overall objective of this thesis is to determine the long-term effects of cholinergic deficiency on molecular hallmarks of AD, as well as elucidating the role of forebrain cholinergic tone in regulating hippocampal-dependent cognitive functions. To address this, the specific aims of this thesis are:

1) Generate and characterize a mouse model of forebrain-specific cholinergic dysfunction.

2) Elucidate the consequences of long-term cholinergic failure on hippocampal neurogenesis and neuropathology.

3) Determine the contribution of forebrain cholinergic signalling in regulating hippocampal-dependent cognitive function.

We hypothesize that forebrain cholinergic tone is required for hippocampal resilience, and distinct forms of cognitive processing.
2. Materials and Methods

2.1 Ethics Statement

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). We followed the ARRIVE guidelines (Kilkenny et al. 2010), hence mice were randomized for behavioural tests and the experimenter was blind to the genotype. Animals were housed in groups of three 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 behavioural testing. 3 month-old male mice were used for behavioural studies.

2.2 Animals

Generation of VAChT\textsuperscript{flox/flox} mice was previously described (Martins-Silva et al. 2011). VAChT\textsuperscript{Nkx2.1-Cre-flox/wt} mice were generated by crossing VAChT\textsuperscript{flox/flox} (mixed C57BL/6J x 129/SvEv background, backcrossed to C57BL/6J for five
generations) with the Nkx2.1-Cre mouse line (C57BL/6J-Tg(Nkx2-1-cre)2Sand/J), purchased from The Jackson Laboratory (JAX stock no. 008661). Offspring was then intercrossed to generate VACHT\textsuperscript{Nkx2.1-Cre-flox/flox} mice. VACHT\textsuperscript{Nkx2.1-Cre-flox/flox} mice and littermate controls used in this study were generated by crossing VACHT\textsuperscript{Nkx2.1-Cre-flox/flox} to VACHT\textsuperscript{flox/flox}. The reporter mouse line Nkx2.1\textsuperscript{(td-Tomato)} was generated by crossing B6.Cg-Gt(Rosa)26Sor\textsuperscript{tm9(CAG-tdTomato)Hze/J} mice (Jackson Laboratory stock no. 007909) with the Nkx2.1-Cre mouse line (JAX stock no. 008661). The reporter mouse line VGLUT3\textsuperscript{(td-Tomato)} was generated by crossing B6.Cg-Gt(Rosa)26Sor\textsuperscript{tm14(CAG-tdTomato)Hze/J} mice (JAX stock no. 007914) with the VGLUT3-Cre mouse line (Tg(Slc17a8-icre)1Edw/SealJ, JAX stock no. 018147). We generated VACHT\textsuperscript{D2-Cre-flox/flox} mice by crossing VACHT\textsuperscript{flox/flox} with the D2-Cre mouse line (D2-Cre mice (Drd2, Line ER44) obtained from the GENSAT). We then intercrossed VACHT\textsuperscript{D2-Cre-flox/wt} to obtain VACHT\textsuperscript{D2-Cre-flox/flox} mice. The D2-Cre-ChAT-flox/flox mouse line was generated by crossing Chat\textsuperscript{tm1(cre/ERT)Nat} with the D2-Cre mouse line (D2-Cre mice (Drd2, Line ER44) obtained from the GENSAT). Cx3cr1\textsuperscript{CreER} mice (JAX stock no. 021160) were intercrossed with B6;129-Gt(ROSA)26Sor\textsuperscript{tm1(CAG-CHRM3/Cit)Ute} mice (JAX stock no. 026220), to obtain Cre recombination; offspring were injected with tamoxifen 75mg/kg intraperitoneally once daily for 5 days. A summary of the mice used and relevant references are highlighted in Table 1.
<table>
<thead>
<tr>
<th><strong>Mouse model</strong></th>
<th><strong>Commercial nomenclature</strong></th>
<th><strong>References</strong></th>
</tr>
</thead>
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<td>Cx3cr1$^{\text{CreER-DREADD}}$</td>
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**Table 1. Summary of the mouse models used in this thesis.**
2.3 Immunohistochemistry

Mice were anesthetized with ketamine (100 mg/kg) and xylazine (25 mg/kg) in 0.9% sodium chloride, and then euthanized by transcardiac perfusion: phosphate-buffered saline (PBS, pH=7.4) for 3 minutes and 4% paraformaldehyde for 5 minutes. Brains were harvested and placed in 4% paraformaldehyde in 1× PBS at 4 °C for 4 hours, they were kept at 4 °C until 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 hour. 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). Next, primary antibodies (Table 2) were incubated in blocking buffer overnight at 4 °C. Sections were then washed five times in 1× PBS/0.4% Triton X-100 (10 minutes each). Hoechst 3342 (Life Technologies. Bibco, Carlsbad, CA, USA) (2–5 μg/ml1) and secondary antibodies (Table 2) were diluted in blocking buffer and slices were incubated for 1 hour at room temperature. Sections were visualized by Zeiss LSM 510Meta (Carl Zeiss, Oberkochen, Germany) confocal system (10x, 40x, 63x objective, 488-nm Ar laser and 633-nm HeNe laser were used for excitation of fluorophores).
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<th>Secondary antibody</th>
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<td>anti-488 (1:500) ThermoFisher (catalog #A-11034)</td>
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Table 2. List of primary antibodies, sources, and secondary antibodies used in this thesis as part of the immunohistochemistry protocol.
2.4 Western Blotting

Brain tissues were frozen in a mixture of dry ice and ethanol and were stored at -80°C. To isolate and extract protein, tissues were homogenised in 10mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 1% Triton X-100, and protease inhibitor cocktail (Sigma-Aldrich, catalog# P8340). Extracts were left on ice for 15 minutes post-homogenization and then centrifuged at 10,000xg for 20 minutes at 4°C. Next, the supernatant was collected and protein concentration was determined using the Bradford assay (Bradford, 1976). 30-60 µg of protein lysates were then separated using SDS-PAGE and transferred onto PVDF membranes. Immuno-blotting was performed as previously described (Martins-Silva et al. 2011; Kolisnyk et al. 2013; Kolisnyk et al. 2013). Antibodies used were anti-VACHT (1:1000) (catalog #139103; Synaptic Systems), anti-Synaptophysin (1:200) (catalog #S5768; Sigma-Aldrich), anti-Doublecortin (DCX; 1:1000) (catalog #18723; Abcam), anti-AT180 (1:1000) (catalog #EN-MN1040; ThermoFisher), and anti-β-actin (catalog #ab49900; Abcam). Band intensities were quantified using FluoroChemQ software (Thermo Fisher Scientific).

2.5 Quantitative 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 Laboratories, Mississauga, Canada) according to the manufacturer's instructions. Total RNA from hippocampal tissue was eluted in 80 µl of Elution solution. Quantification and quality control of RNA in the extracted samples was done by microfluidic analysis using Agilent 2100 Bioanalyzer (Agilent Technologies Inc. Palo Alto, CA, USA). Next, 20 µl of cDNA was synthesized from 500 ng of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Streetsville, Canada) according to the manufacturer's instructions. qPCR was then performed on the cDNA on a CFX-96 Real Time System (Biorad) using iQ SYBR GREEN SUPERMIX (Biorad). PCRs were cycled 40 times after denaturation (95°C for 3 minutes) and a non-template reaction was used as a negative control. β-actin gene expression was to normalize data, and relative quantification of gene expression was performed with the DDCT method.

2.6 Thioflavin S Staining

Thioflavin S staining was performed by incubating mounted sections (30µm) with a series of graded ethanol: 100% for 3 minutes, 95% for 3 minutes, 70% for 3 minutes, 50% for 3 minutes. Next, sections were washed with water, twice for 3 minutes. Next, sections were incubated in a solution of 0.05% Thioflavin S in 50% ethanol, rinsed in ethanol 100% followed by water (Sun et al. 2002).
2.7 Silver staining

Assessment of argyrophilic cells in the hippocampus was done using NeuroSilver™ staining kit II (FD NeuroTechnologies, Inc., Baltimore, MD), which provides detection of degenerating neurons, including neuronal somata, axons, and terminals.

2.8 Stereotaxic injections of adeno-associated virus (AAV)

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 AAV8-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 minutes to stabilize. After stabilization, a 0.2 µl per minute infusion was performed using a micropump followed by a 30 minutes 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 behavioural testing to allow transgene expression.
2.9 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 using a feedback controlled rectal thermometer and kept between 36.5°C and 37°C using a heating pad. Stimulating electrodes were placed into stratum radiatum at P 1.8, L 2.3 or P 2.5, L 2.4 (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 x 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 minutes (coefficient of variation (SEM/mean) of the sink slopes <0.05), a high-frequency tetanus (100 Hz for 1s) was delivered at 2-3 x threshold intensity, and the response was measured for 120 minutes 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.

2.10 Open Field Locomotion

Measurement of spontaneous locomotor activity in a novel environment was performed as previously described (Pierce and Kalivas 2007; de Castro et al. 2009). Mice were put in an open field box (20 cm x 20 cm width, 30 cm length), and movement was tracked and recorded via VersaMax Animal Monitoring System (AccuScan Instruments Inc., Columbus, OH). Mice were allowed to freely move in the novel environment for 120 minutes. Movement (cm) per 5-minute interval was obtained by VersaMax analysis, which analyses infrared beam breaks.

2.11 Metabolic Assessment

To assess metabolic activity in mice, we measured oxygen consumption, carbon dioxide production, respiratory exchange ratio, food and water intake, and physical activity using the Comprehensive Lab Animal Monitoring System (CLAMS) using Oxymax software (Columbus Instruments, Columbus, OH). Mice were individually housed in the metabolic cages at 24°C and provided with free access to powdered standard rodent chow and water. Following an initial 16-hour habituation period, measurements were taken every 10 minutes for 24 hours (12-
hour light/12-hour dark cycle). Volume of oxygen consumption and carbon dioxide production measurements were normalized to body mass. For total activity, ambulatory activity, and inactivity periods (sleep) the Opto-M3 Activity Monitor and Oxymax algorithms (Columbus Instruments, OH) were used.

2.12 Morris Water Maze

The spatial version of the MWM was performed as previously described (Vorhees and Williams 2006; Martyn et al. 2012; Kolisnyk et al. 2013). Testing was performed in a 1.5-meter-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 four arbitrarily defined quadrants, and spatial cues were distributed around the pool (posters, streamers, and plastic props). Mice were given four 90-second trials for the duration of 4 days to find the hidden platform, with an inter-trial interval (ITI) of 15 minutes. 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 seconds were gently guided to the platform. On the fifth day, spatial memory recall was tested by a 60-second 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-second trials for 4 days. On the fifth day, the animals were given a 60-second probe trial. Data were analyzed using ANY-Maze video tracking software (Stoelting Co.).
The classification of search strategies mice employed during training was defined as previously described (Garthe et al. 2009, Wolfer et al. 2000). 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.

The cued variation of the MWM was performed as described previously (Vorhees & Williams, 2006). The cued variation was 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.

2.13 Rotarod

To measure motor coordination, an accelerating rotarod (San Diego Instruments; San Diego, CA) was used as previously described (Prado et al. 2006; de Castro et al. 2009). Prior to testing, mice were allowed to habituate to the rod for 5 seconds. During the first 100 seconds of testing, the rotarod rotates at 5 revolutions per minute (RPM), and then accelerates to 15 RPM for the next 100 seconds, then 25-RPM for the next 100 seconds, and 35 RPM for the final 100 seconds. Mice were given ten trials with a 10-minute inter-trial interval in their home cage. The trial ended when a mouse falls off the rod or completed 400 seconds on the rod. The rod was cleaned with 70% ethanol. On the following day, the conditions remained the same, except mice were placed on the accelerating rotarod for four trials.

2.14 Wire Hang Test

The wire hang test was performed as previously described (Sango et al. 1996; Prado et al. 2006; Deacon 2013). Each mouse was placed on a wire-grid; the experimenter then slowly inverted and suspended the wire approximately 40 cm above a foam mat. The amount of time from the inversion of the wire till the
mouse fell from the wire was measured, with a 60 second limit. Each mouse was given an inter-trial interval of 30 seconds in its home cage.

### 2.15 Grip Strength

Forelimb grip strength was assessed using a grip strength meter (Columbus Instruments, Columbus, OH) (Prado et al. 2006). Each mouse grasped the pull bar of the apparatus with their forelimbs only and the experimenter gently pulled the mouse backwards by the tail maintaining mouse body horizontally. The force applied to the bar prior to the release of the grasp was recorded. The average of 3 measurements were recorded and an inter-trial interval of 30 seconds was given to each mouse in their home cage.

### 2.16 Forced swim test

To assess depressive-like behaviour, mice were subjected to the forced swim test (Martyn et al. 2012). Mice were placed in the testing room to acclimate to the environment for one hour. Next, each mouse was placed in a 2 L beaker with 1.8 L of water (25-27°C) for 6 minutes. A video camera was placed above the beaker and immobility time, and distance swam were recorded and analyzed using ANY-Maze software (Stoelting Co., IL). The first minute of activity was not used for the analysis, that is, graphs show information from 2-6 minutes of the test. Water was changed after every 2 mice completed testing.
2.17 Tail Suspension Test

Mice were assessed for depressive-like behaviour as previously described (Martyn et al. 2012). Mice were suspended from a metal bar 30 cm above a table by their tail, using a masking take placed at the tip of the tail, for 6 minutes. A video camera was placed in front of the rod facing the suspended mouse. Sessions were recorded and immobility time and immobility episodes from 2-6 minutes were recorded using ANY-Maze software (Stoelting Co., IL).

2.18 Paired Associates Learning Training Phase

Prior to training, mice were food restricted until they reached approximately 85% of their original weight. Training of the animals to the PAL task was previously described (Figure 4) (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 minutes 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 150µl 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 minutes training session was done for the
next 2 days until mice completed 36 trials in 60 minutes (Habituation; phase 1). Mice were then trained to associate the reward with a 30 second 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 in order 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 minutes for one day. Next, mice were required to touch the stimulus that is displayed randomly in one of the 3 windows in order 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 one 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 minutes for one 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 i.e. one of the blank screens, it was presented with a 5-second 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 minutes for 2 consecutive days. Next, both experimental groups were subjected to acquisition training, where two
stimuli were displayed at the same time during a trial. One was in the correct location (S+) and the other was in the incorrect location (S-). Mice were required to touch the correct stimulus (S+) presented on one of the three screens to complete a trial and receive the reward. In this acquisition phase, mice were on an unpunished version where 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 one session (one day). All mice from both experimental groups were able to reach criterion in acquisition training. A summary of the training phases is shown in Figure 4.
Figure 4. Brief description of the parameters and criterion for the operant conditioning and pretraining phases prior to being tested on the PAL task.

(A) Habituation; phase 1, (B) Initial touch; phase 2, (C) Must touch; phase 3, (D) Must initiate; phase 4, (E) Punish incorrect; phase 5, (F) dPAL acquisition.
2.19 Paired Associates Learning

After successfully completing the training phase, mice were placed on a PAL task (dPAL), which involves a different stimulus being presented in each trial (Figure 5). 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 six possible trial types and three 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 10s time-out and the chamber light was activated for 10s, acting as an indication for an incorrect response for the mouse. After 10s, 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 towards 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.
Figure 5. Image depicting the six possible trial types that were presented during the dPAL task. (A) The flower image is S+ when presented in screen 1, (B) The spider image is S+ when presented in screen 3, (C) the airplane image is S+ when presented in screen 2.
2.20 Two-Choice Location Discrimination task

Operant conditioning and pretraining on the touchscreen system was performed as described in the PAL training, except a mask containing 5 windows was used, instead of 3. Next, mice were put on the 2-choice location discrimination task. Mice were required to touch one of two illuminated screens (e.g. S+ is in the most left screen) until a criterion of 9/10 consecutive correct trials was achieved. After criterion was achieved the separation of the illuminated screens was presented with either a high degree of separation (easy; 3 blank screens separating the illuminated screens), or a low degree of separation (hard; 1 blank screen separating the illuminated screens). Mice were tested on this task for 10 days. Next, the designated correct location of the screen was switched to the other location (e.g. S+ is now in the most right screen). Mice were tested on this task for 10 days. Groups of mice were counterbalanced across the separations. Data were analyzed by averaging the number of trials required by each mouse on each separation (easy and hard) during 20 days of testing.

2.21 Quantification of labeled cells

After completion of training phases, 4 mice from each testing group were injected with BrdU 50mg/kg i.p. once daily for 3 days. Quantification of immunolabeled cells in the dentate gyrus of the hippocampus was performed as previously described (Clelland et al. 2009). One-in-twelve series of 40 \( \mu \)m brain sections
from each animal was immunostained (see section 2.3 immunohistochemistry). Using confocal microscopy, immunolabeled cells in the dentate gyrus were counted using 63x objective (Zeiss LSM 510Meta, Carl Zeiss, Oberkochen, Germany). Estimation of the total number of positively labeled cells per hippocampus was obtained by multiplying the number by 12.

2.22 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 binomial distribution (Anders and Huber 2010). Datasets are available on ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-3897.
2.23 Statistical Analysis

All data are expressed as mean ± SEM. Graphpad and Sigmasstat 3.5 software were used for statistical analysis. Comparison between two experimental groups was done with Student’s t-test. When several experimental groups or treatments were analyzed, two-way analysis of variance (ANOVA) or two-way ANOVA with repeated measures (RM) were used as required. When appropriate, a Bonferonni post-hoc analysis test was used.
Chapter 3

3. Results

3.1 Generation of forebrain-specific cholinergic deficient mouse model

3.1.1 Targeting forebrain cholinergic neurons in the mouse brain

As we intended to use Nkx2.1-Cre transgenic mice to generate a mouse model with VACHT gene deletion specifically from forebrain cholinergic neurons, it was important to determine whether the Nkx2.1 promoter would in fact drive Cre expression in forebrain cholinergic neurons and also if cholinergic neurons from other brain areas would not express Cre. Nkx2.1 is a homeodomain-containing transcription factor that is expressed in mice at embryonic day E9 and is restricted to the ventral pallidal telencephalon (Sussel et al. 1999; Xu et al. 2008). To assess the brain areas where Nkx2.1-Cre is expressed and co-localizes with cholinergic neurons, we used a Rosa-26-tdTomato reporter mouse line. In this reporter mouse line, the Rosa26 locus expresses tdTomato, a DsRed fluorescent protein variant. Upon recombination, Cre excises the stop codon, which is
flanked by loxP sequences upstream from the translational initiation codon of tdTomato, allowing translation of the protein (Figure 6A). We crossed Rosa-26-tdTomato mice with Nkx2.1-Cre mice and performed immunohistochemistry analysis to evaluate the localization of Cre-mediated expression in cholinergic neurons. Co-localization analysis of choline transporter (CHT1) expression, and tdTomato expression indicated that these two proteins are present in the same cells mainly in the basal forebrain (95% co-localization) and the striatum (38% co-localization), while no co-localization was observed in the Mo5 motor nucleus or the pedunculopontine nucleus (PPN) (Table 3; Figure 6B-D). These findings indicated that Nkx2.1-Cre mice could be utilized to eliminate floxed genes specifically from forebrain cholinergic neurons.

Using the same genetic approach, we investigated if the vesicular glutamate transporter (VGLUT3) promoter drives Cre expression in forebrain cholinergic neurons. We crossed Rosa-26-tdTomato mice with VGLUT3-Cre mice and assessed co-localization with ChAT. We observed that there was approximately 82% co-localization between ChAT and tdTomato in the basal forebrain, 80% in the striatum and 2% co-localization in the PPN (Table 4; Figure 7A). Although the VGLUT3 promoter also showed to drive Cre expression in forebrain cholinergic neurons, these results showed that Cre expression was observed in a higher population of cholinergic basal forebrain neurons when we used the Nkx2.1 promoter. Thus, we decided to use the Nkx2.1-Cre to target forebrain cholinergic neurons in our studies.
Figure 6. Nkx2.1-Cre drives Cre expression in forebrain cholinergic neurons. (A) Scheme illustrating Nkx2.1-Cre transgene and reporter system for Cre-mediated recombination in Rosa26-tdTomato mice. (B) Expression pattern of Cre detected by tdTomato fluorescence in the brain of Nkx2.1-Cre;tdTomato mice (Scale bar, 2 mm). (C-D) Expression of Cre detected by tdTomato fluorescence and co-localization analysis with CHT1 in different regions in the brain of Nkx2.1-Cre;tdTomato mice. B.F= basal forebrain, PPN= pedunculopontine nucleus, Mo5= motor trigeminal nucleus (Scale bar, 50 μm)
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Table 3. Percentage of colocalization between cells that express CHT1 and Cre-mediated tdTomato fluorescence in different regions of the brain of NKx2.1-Cre;tdTomato mice.
Figure 7. Expression of Cre detected by tdTomato fluorescence and co-localization analysis with ChAT in different regions in the brain of VGLUT3-Cre;tdTomato mice. NbM = Nucleus Basalis Meynert, PPN = pedunculopontine nucleus (Scale bar, 50 µm)
Table 4. Percentage of colocalization between cells that express ChAT and Cre-mediated tdTomato fluorescence in different regions of the brain of VGLUT3-Cre;tomato mice.

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3.1.2 *Elimination of cholinergic tone in forebrain projection neurons*

Decreased VAChT levels severely compromises packaging of ACh into synaptic vesicles and subsequently decrease ACh release by nerve terminals (Prado *et al.* 2006; de Castro *et al.* 2009). Similarly, overexpression of VAChT increases ACh release (Song *et al.* 1997; Kolisnyk *et al.* 2013). Genetic targeting of either VAChT (Guzman *et al.* 2011; Martyn *et al.* 2012), or 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). We bred a mouse line containing the floxed allele of VAChT (VAChT<sup>flox/flox</sup>) with an Nkx2.1-Cre transgenic line to obtain mice with VAChT deletion restricted to the forebrain: VAChT<sup>Nkx2.1-Cre-flox/flox</sup> mice. Using immunohistochemistry, we assessed VAChT expression in the hippocampus of VAChT<sup>flox/flox</sup> and VAChT<sup>Nkx2.1-Cre-flox/flox</sup> littermates. Our analysis revealed decreased VAChT immunoreactivity in the hippocampus of VAChT<sup>Nkx2.1-Cre-flox/flox</sup> mice compared to controls (Figure 8A-B). Moreover, 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<sup>Nkx2.1-Cre-flox/flox</sup> mice (Figure 8C-E). In contrast, VAChT levels remained unchanged in the brainstem of VAChT<sup>Nkx2.1-Cre-flox/flox</sup> compared to controls ($t_{(4)}=1.040$, $p=0.3571$, Figure 8F).
Figure 8. VChT deletion from forebrain projection neurons. (A) Representative 3-dimensional reconstructed Z stack immuno-fluorescence images of VChT (green) and Hoechst (blue) in the CA1 and CA3 regions of the hippocampus in VChT$^{flox/flox} (n=3)$ and (B) VChT$^{Nkx2.1-Cre-flox/flox}$ mice (n=3) (Scale bar= 100µm). VChT expression and quantification from Western blots in the whole cortex (C), hippocampus (D), striatum (E), brainstem (F) in VChT$^{flox/flox}$ (clear bars) and VChT$^{Nkx2.1-Cre-flox/flox}$ (dark bars) mice. VChT expression was normalized to synaptophysin (n= 4, data are mean ± SEM. **P<0.01).
3.2 Determining the contributions of cholinergic signalling for hippocampal health

3.2.1 Long-term deficient cholinergic tone leads to age-dependent protein aggregation

The pathogenic spread of protein aggregation in neurons is a hallmark of several neurodegenerative diseases, including AD (Walsh and Selkoe 2016). To investigate the consequences of long-term cholinergic deficiency on hippocampal neuropathology, we evaluated pathology in the hippocampus of young (3-6 month old) and old (11-14 month old) VACHT<sup>Nkx2.1-Cre-flox/flox</sup> mice. Aged VACHT-deficient mice exhibited an increase in hippocampal Thioflavin-S positive aggregates compared to age-matched controls (Figure 9A, 11-14 month old mice). High-power micrographs revealed intracellular localization rather than extracellular deposits of Thioflavin-S aggregates (Figure 9A). Interestingly, 3-6 month old VACHT<sup>Nkx2.1-Cre-flox/flox</sup> mice did not present this alteration (Figure 9B). Moreover, control experiments with aged Nkx2.1-Cre mice failed to show any Thioflavin-S positive aggregates (Figure 9C). These results suggest that long-lasting decrease in cholinergic signalling leads to intracellular deposits of protein aggregates.
Figure 9. Aged VACHT-deficient mice present protein aggregates in the hippocampus in an age-dependent manner. (A) Representative images of Thioflavin-S staining of the hippocampus of aged (11-14 month old) controls and VACHT<sup>NKx2.1-Cre-flox/flox</sup> mice (n=5, Scale bar, 100µm and 20 µm for the inset). (B) Very mild Thioflavin-S positive staining in 3-6 month old VACHT<sup>NKx2.1-Cre-flox/flox</sup> mice. (C) Absence of Thioflavin-S positive staining in 11-14 month old C57BL/6J-NKx2.1-Cre mice, compared to 11-14 month old VACHT<sup>NKx2.1-Cre-flox/flox</sup> mice (n=3).
3.2.2 Deficient cholinergic tone leads to hippocampal tau hyperphosphorylation

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 (Lim et al. 2008). Immunofluorescence imaging revealed a robust increase in AT180 immunoreactivity in the hippocampus of VACHT\textsuperscript{Nkx2.1-Cre-flox/flox} mice compared to aged-matched controls (Figure 10A 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 (aa 7-9) and C-terminal (313-333) amino acid sequences of tau, which is one of the earliest alterations of tau in AD (Wolozin et al. 1986; Weaver et al. 2000). Immunostaining with MC1 revealed positive immunoreactivity in the hippocampus of aged cholinergic-deficient mice, but not in age-matched controls (Figure 10B). In agreement with the
immunofluorescence data, hippocampal extracts of VACHT\textsuperscript{Nkx2.1-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 10C and F). On the other hand, total tau and pTauS262 levels were unmodified in VACHT-deficient mice (Figure 10D-F). Taken together, our data indicate that deletion of hippocampal VACHT induces hyperphosphorylation of tau and leads to tau pathological conformation as detected by MC1, both of which are consistently observed in AD.
Figure 10. VACHT-deficient mice present hippocampal tauopathy. (A) Phosphorylated Tau levels monitored by immunolabeling with phosphorylation-dependent antibodies specific to pT231. Representative images of pT231 and Hoechst labeling in the hippocampus of 11-14 month old controls (left) and VACHT<sup>Nkx2.1-Cre-flox/flox</sup> (right) mice. (n=3, Scale bar, 100µm). (B) Representative images of MC1 and Hoechst labeling in the CA1 region of the hippocampus of controls (left) and VACHT<sup>Nkx2.1-Cre-flox/flox</sup> (right) mice. (n=3, Scale bar, 100µm). (C) Western blot analysis of controls (VACHT<sup>flox/flox</sup>) and VACHT<sup>Nkx2.1-Cre-flox/flox</sup> aged (11-14 month old) hippocampal samples for tau using phosphorylation-dependent anti-tau antibodies pT231, (D) Ser 262 and (E) for total Tau protein expression. (F) Quantification of Western blots. pT231, Ser 262, and total tau expression were normalized to actin (n= 4, data are mean ± SEM. **P<0.01).
3.2.3 Long-term hippocampal cholinergic signalling is required for target cell maintenance

In AD, an increase in inflammatory markers' expression and apoptosis are consistently seen (DeKosky and Scheff 1990; Smale et al. 1995; Rogers and Shen 2000). To test whether decreased cholinergic signalling affects vulnerability of target cells in the hippocampus we used silver staining and activation of caspase-3 analysis in hippocampal slices. Aged (11-14 months old) VACHT-deficient mice presented intensified silver staining compared to controls; this increased silver staining was not observed in 3-6 months old VACHT-deficient mice (Figure 11A-B), suggesting that long-lasting decrease in cholinergic signalling may increase the vulnerability of hippocampal neurons. In addition, activated caspase-3, a marker of apoptosis, was augmented in young (3-6 months old) VACHT\(^{Nkx2.1-Cre-\text{floxflox}}\) mice compared to controls (Figure 11C). However, aging (11-14 months old) significantly increased the number of apoptotic cells in VACHT-deficient mice when compared to age-matched controls (Figure 11C).

To test whether cholinergic signalling affects inflammatory responses in target cells in the hippocampus, we assessed microglial activation in VACHT\(^{Nkx2.1-Cre-\text{floxflox}}\) mice and controls (Figure 11D). We found that the expression of CD-68, a marker of activated microglia was increased in the hippocampus of VACHT\(^{Nkx2.1-}\)
Cre-flox/flox mice compared to controls (Figure 11D). Moreover, transcript levels of inflammatory markers: Il-1β and IL-6 were up regulated in the hippocampus of VACHT-deficient mice (Figure 11D).
Figure 11. VACHT-mutant mice show increased neuronal vulnerability in the hippocampus. (A) Representative images of silver staining in the CA1 region of young (3-6 months) and aged (11-14 months) mice. Scale bar, 100µm. (B) Quantification of silver stain-positive cells between young and aged hippocampi of controls (VACHT<sup>flox/flox</sup>, gray bars) and VACHT<sup>Nkx2.1-Cre-flox/flox</sup> (red bars). (n=5, data are mean ± SEM. **P<0.01). (C) Representative immunofluorescence images for activated-caspase 3 labeling in the hippocampi of young (Top) and aged (Bottom) mice. Quantification of activated caspase-3 immunoreactivity in young and aged hippocampi of controls (VACHT<sup>flox/flox</sup>, gray bars) and VACHT<sup>Nkx2.1-Cre-flox/flox</sup> (red bars). (D) Immunofluorescence imaging and quantification showing CD-68 immunoreactivity in the hippocampus of aged VACHT<sup>Nkx2.1-Cre-flox/flox</sup> mice as well as levels of IL-1β transcripts as measured by qRT-PCR (t<sub>(10)</sub>=2.312, p=0.0434, n=6) and IL-6 transcripts as measured by qRT-PCR (t<sub>(10)</sub>=2.882, p=0.0204). (Data are mean +/- S.E.M., *P<0.05, n=6 for qPCR analysis). (n= 3 for immunofluorescence, data are mean ± SEM. *P<0.05, **P<0.01, Scale bar, 100µm)
3.2.4 Generating a mouse line to investigate cholinergic microglial activation

Based on our findings of increased neuroinflammation due to decreased cholinergic tone, it seemed of importance to investigate how cholinergic tone can regulate microglial activation. One possibility to explain these results is that chronic cholinergic modulation of microglia, via activation of muscarinic or nicotinic receptors maintains microglial inflammatory responses in check. In order to start to address this possible modulation, we have generated a new mouse model in which muscarinic modulation can be regulated exclusively in microglia. To do that, we used the Cre technology to generate a mouse that expresses the DREADD hM3Dq specifically in microglia. To generate these mice, we intercrossed Cx3cr1\(^{CreER}\) mice (Parkhurst et al. 2013) with B6;129-Gt(ROSA)26Sor\(^{tm1(CAG-CHR33Cit)Ute}\) (Gq-DREADD) mice (Roth 2016). Gq-DREADD mice carry HA-hM3Dq-pta-mCitrine in the ROSA locus, with a loxP-flanked STOP codon to prevent transcription of the downstream HA-hM3Dq-pta-mCitrine gene. Cx3cr1\(^{CreER}\) mice express tamoxifen-inducible Cre recombinase under the control of the Cx3cr1 promoter, which was previously shown to target myeloid cells (Parkhurst et al. 2013). Upon tamoxifen-induced Cre expression, the flanked STOP codon would be excised and expression of HA-tagged hM3Dq should be allowed, specifically in Cx3cr1-positive cells. To specifically target
microglia from the Cx3xr1-positive cells, we waited 30 days post-tamoxifen injection prior to examine the brains of these mice. This delay was given to account for unwanted recombination of peripheral myeloid Cx3xr1-positive cells. Peripheral Cx3xr1 cells would initially undergo recombination, but because of their high turnover rate (van Furth and Cohn 1968), non-recombined cells would replace them in the absence of tamoxifen in their body (Parkhurst et al. 2013). On the contrary, microglia have a low turnover rate (Lawson et al. 1992) and therefore, the recombined cell population should be maintained even after tamoxifen dissipates (Parkhurst et al. 2013).

To determine whether we have generated mice expressing Gq-DREADD in microglia, we used immunohistochemistry to probe for anti-HA, and assessed the percentage of co-localization with IBA1 (microglia-specific marker) in 30 µm sections of the hippocampus of mice treated with tamoxifen (Figure 12A). Our studies revealed approximately 87% co-localization between anti-HA positive cells and IBA1-positive cells in the hippocampus of these mice (Figure 12B). We observed no labeling with HA in sections from mice that were not treated previously with tamoxifen. This high degree of co-localization suggests that this model may be utilized for assessing the consequences of activation of central inflammatory responses using chemogenetics. Experiments with this new mouse model in the laboratory confirmed that activation of microglia in culture with clozapine-N-Oxide (CNO) specifically activates intracellular calcium signalling only in microglia previously treated with tamoxifen (not shown).
Figure 12. Expression of hM3Dq in microglia. (A) Reconstructed 3D Z-Stack immunofluorescence images of Iba1 (green) and anti-HA (red) in the prefrontal cortex region of the mouse. (B) Representative immunofluorescence images of Hoechst (blue), Iba1 (green), and anti-HA (red). Graph indicates co-localization analysis in the hippocampi of Cx3cr1<sup>CreER-DREADD</sup> mice. (n= 4, Scale bar, 100µm)
3.2.5 Hippocampal cholinergic deficits starting in adulthood

Our experiments suggest that long-term loss of cholinergic signalling leads to increased neuronal vulnerability and apoptosis in the hippocampus. However, because VAChT\textsuperscript{Nkx2.1-Cre-flox/flox} mice show abolished cholinergic tone from embryonic stages it was important to determine if the effects observed were not due to developmental deficits. To investigate that, we evaluated pathology in the hippocampus of adult VAChT\textsuperscript{flox/flox} mice that received stereotaxic injections of AAV8-GFP-Cre in the medial septum and vertical limb of the diagonal band (MS/VDB). Aged (12-16 months old) VAChT\textsuperscript{flox/flox} mice received stereotaxic injections of AAV8-GFP-Cre (n=5) or AAV8-GFP (n=3) virus to their medial septum and vertical limb of the diagonal band (MS/VDB) as described (Al-Onaizi et al. 2016) and 6 months post-injections, we assessed VAChT levels and pathology in the hippocampus (Figure 13A-B).

We found that hippocampal VAChT levels were decreased approximately 50\% \((t_{(6)}=2.925, p=0.0265, \text{Figure 13A-B})\) in AAV8-GFP-Cre injected mice, compared to controls. Analysis of AT180 tau epitope (T231/S235) immunoreactivity in the hippocampus in both groups showed no differences in labeling (Figure 13C). Silver deposits showed a tendency to be increased in AAV8-GFP-Cre injected mice, but without overall significant statistical difference \((t_{(6)}=1.983, p=0.0946, \text{Figure 13D})\). In contrast, activated caspase-3, a marker of apoptosis, was augmented in aged VAChT\textsuperscript{flox/flox} mice injected with AAV8-GFP-Cre compared to AAV8-GFP injected VAChT\textsuperscript{flox/flox} controls \((t_{(6)}=2.659, p=0.0376, \text{Figure 13E-F})\).
Figure 13. Neuropathology analysis in AAV8-GFP-Cre injected VACHflox/flox mice. (A) Representative images of VACH immunoreactivity in the hippocampus and (B) quantification of aged VACHflox/flox GFP injected controls and VACHflox/flox Cre injected mice. (C) Representative images of pT231 and Hoeschst labeling in the hippocampus of VACHflox/flox GFP injected controls and VACHflox/flox Cre injected mice (Scale bar, 100µm). (D) Representative images and quantification of silver staining in the CA1 region VACHflox/flox GFP injected controls and VACHflox/flox Cre injected mice (Scale bar, 100µm). (E) Representative immunofluorescence images and (F) quantification for activated-caspase 3 labeling in VACHflox/flox GFP (n=3) injected controls and VACHflox/flox Cre injected mice (n=5). (Data are mean ± SEM. *P<0.05, **P<0.01, Scale bar, 100µm)
3.2.6  *Hippocampal long-term potentiation in mice with deficient cholinergic tone*

Synaptic plasticity is a critical component of hippocampal function and is thought to form the cellular basis of learning and memory (Gu and Yakel 2011; Kanju et al. 2012). Mice with reduced VACHT levels present deficits in LTP in hippocampal slices in vitro (Martyn et al., 2012). Thus, we aimed to determine whether these deficits are reproducible *in vivo* in VACHT$^{Nkx2.1-Cre-flox/flox}$ mice. We examined LTP of the synapse of the Schaffer collaterals on hippocampal CA1 neurons in anaesthetised mice. VACHT$^{Nkx2.1-Cre-flox/flox}$ mice showed decreased LTP which lasted about 90 minutes post-tetanus delivery while LTP in VACHT$^{flox/flox}$ mice was maintained for 120 minutes ($t_{(9)}=3.911$, $p=0.0036$, Figure 14A-B). This indicates that the lack of cholinergic signalling disturbs synaptic plasticity in hippocampal CA1 area *in vivo*. 
**Figure 14. 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\textsuperscript{flox/flox} (clear squares, n=5) and VACHT\textsuperscript{Nkx2.1-Cre-flox/flox} (dark circles, n=6) mice. Baseline was monitored for 30 minutes prior to tetanus delivery (t=0), and post-tetanic response was monitored for 120 minutes. A 1-second 100 Hz train, delivered at 2-3 times the threshold intensity (arrow), induced higher and more prolonged potentiation in VACHT\textsuperscript{Nkx2.1-Cre-flox/flox} mice than VACHT\textsuperscript{flox/flox} controls. Insets show representative current sink time response taken at 80 minutes (red traces), overlaid on the pre-tetanus baseline response (black traces), from each genotype. (B) Normalized excitatory sink slope averaged across 30 minute time intervals (mean ± SEM) in VACHT\textsuperscript{flox/flox} and VACHT\textsuperscript{Nkx2.1-Cre-flox/flox} mice, with significant difference between mouse groups at 90-120 minutes (t(9)=3.911, p=0.0036)
3.2.7 *Cholinergic tone is required for adult hippocampal neurogenesis*

Cholinergic signalling has been implicated in modulating neurogenesis (Cooper-Kuhn *et al.* 2004; Kaneko *et al.* 2006), and neurogenesis contributes to hippocampal function (Shors *et al.* 2001). Initial genome-wide transcriptome analysis indicated that genes related to neurogenesis were differentially expressed in the hippocampus of VACHT<sup>Nkx2.1-Cre-flox/flox</sup> mice compared to control mice (not shown; Benjamin Kolisnyk). In agreement, qPCR analysis confirmed changes in expression of many neurogenesis-related genes (Figure 15A). Therefore, we sought to investigate if neurogenesis was altered in the hippocampi of VACHT-deficient mice. A key step in neurogenesis is the proliferation of cell precursors in the dentate gyrus, thus we analyzed the immunoreactivity of the proliferation marker Ki67 in the dentate gyrus of VACHT<sup>Nkx2.1-Cre-flox/flox</sup> mice (Figure 15B). Quantification of Ki67 expression revealed a decrease in the number of proliferating cells in the SGZ of the hippocampus of VACHT<sup>Nkx2.1-Cre-flox/flox</sup> mice in comparison to controls ($t_{(6)}=2.455$, $p=0.0433$) (Figure 15C).

Moreover, immunoreactivity of the neuroblast-specific marker, doublecortin, revealed decreased expression in the SGZ of the hippocampus of VACHT<sup>Nkx2.1-Cre-flox/flox</sup> mice compared to controls (Figure 15D). Quantification using
immunoblot analysis showed that doublecortin expression was decreased ($t_{(6)}=3.838$, $p=0.0086$) in VACHT-deficient mice, suggesting that decreased cholinergic tone affects neuroblast formation in the hippocampus (Figure 15E). Furthermore, 24-hour BrdU labeling in VACHT-deficient mice revealed a significant decrease in the number of BrdU-positive cells in the SGZ of the hippocampus, compared to controls ($t_{(6)}=5.174$, $p=0.0021$) (Figure 15F-G). Together, these findings suggest that hippocampal cholinergic tone may be critical in regulating proliferation as well as differentiation of neuronal precursor cells.
Figure 15. VACHT<sup>Nkx2.1-Cre-flox/flox</sup> mice show impaired neurogenesis in the subgranular zone of the hippocampus. (A) Transcript levels measured by qPCR of neurogenesis-related genes identified by whole-transcriptome analysis. (B) Immunoreactivity and (C) quantification of the number Ki67-positive cells in the dentate gyrus of VACHT<sup>Nkx2.1-Cre-flox/flox</sup> and controls. (D) Doublecortin immunoreactivity in the dentate gyrus of VACHT<sup>Nkx2.1-Cre-flox/flox</sup> and controls. (E) Immunoblot analysis and quantification of doublecortin expression in the hippocampus of VACHT<sup>Nkx2.1-Cre-flox/flox</sup> and controls. (F-G) BrdU expression and quantification in the dentate gyrus of controls and VACHT<sup>Nkx2.1-Cre-flox/flox</sup> mice. (n=4, data are mean ± SEM. *P<0.05, **P<0.01, ***P<0.0001).
3.3 Behavioural characterization of forebrain-specific VACht-deficient mice

3.3.1 Neuromuscular analysis of VACht\textsuperscript{NKx2.1-Cre-flox/flox} mice

In order to understand whether cholinergic deficiency leads to cognitive deficits, we examined several behavioural parameters in the VACht-deficient mice. To investigate if deletion of VACht has consequences on neuromuscular performance, we performed a battery of motor tasks. VACht\textsuperscript{flox/flox} and VACht\textsuperscript{NKx2.1-Cre-flox/flox} mice show no differences in performance in the wire-hang test ($t_{(10)}=1.0, p=0.341$, Figure 16A) and the grip force test ($t_{(10)}=0.124, p=0.903$, Figure 16B). In addition, we analyzed motor skill learning using the rotarod. Performance of VACht\textsuperscript{NKx2.1-Cre-flox/flox} mice was very similar to VACht\textsuperscript{flox/flox} control mice, (Two-way RM ANOVA shows an effect of days $F_{(13,65)}=5.821$, $p<0.0001$, no genotype effect, $F_{(1,5)}=0.310$, $p=0.6014$, and no interaction $F_{(13,65)}=0.652$, $p=0.801$, Figure 16C), suggesting that forebrain cholinergic deficiency does not have major impact on neuromuscular performance.

One potential limitation regarding Cre recombinase-expressing mouse lines is that the Cre transgene might lead to non-specific effects aside from excision of the target gene. Thus, we assessed the behavioural phenotype of mice that only
express Cre, but do not contain the targeted floxed alleles (Nkx2.1-Cre mice). Mice were initially assessed on neuromuscular function. In the wire-hang test, Nkx2.1-Cre mice were no different from wild-type C57BL/6 littermates in time spent hanging upside down from a wire netting ($t_{(10)}= 0.447, p=0.664$, Figure 17A). Similarly, Nkx2.1-Cre mice displayed no impairments in the grip force test, compared to controls ($t_{(10)}=0.255, p=0.803$, Figure 17B). We also analyzed motor skill learning using the rotarod. Performance of Nkx2.1-Cre mice was very similar to C57BL/6 control mice (Two-way RM ANOVA shows an effect of days $F_{(13,91)}=5.149, p<0.0001$, no genotype effect, $F_{(1,7)}=0.084, p=0.779$, and no interaction $F_{(13,91)}=0.662, p=0.7938$, Figure 17C).
Figure 16. Neuromuscular testing on $\text{VACHT}^{\text{flox/flox}}$ and $\text{VACHT}^{\text{Nkx2.1-Cre-flox/flox}}$ mice. (A) Wire-hang test; $\text{VACHT}^{\text{flox/flox}}$ (white) and $\text{VACHT}^{\text{Nkx2.1-Cre-flox/flox}}$ (black) mice. (B) Grip force; $\text{VACHT}^{\text{flox/flox}}$ (white) and $\text{VACHT}^{\text{Nkx2.1-Cre-flox/flox}}$ (black) mice. (C) Performance in the rotarod; $\text{VACHT}^{\text{flox/flox}}$ (white, n=8) and $\text{VACHT}^{\text{Nkx2.1-Cre-flox/flox}}$ (black, n=8) mice.
Figure 17. Neuromuscular testing on C57BL/6 littermates and Nkx2.1-Cre mice. (A) Wire-hang test; WT littermate controls (C57BL/6 white, n=6), and Nkx2.1-Cre (gray, n=6). (B) Grip force; WT littermate controls (C57BL/6, white, n=6), and Nkx2.1-Cre (gray, n=6). (C) Performance in the rotarod; WT littermate controls (C57BL/6, white, n=6), and Nkx2.1-Cre (gray, n=6). (B) Grip force; (C57BL/6 white squares, n=8), and Nkx2.1-Cre (gray circles, n=8).
3.3.2  **Spontaneous locomotor activity in VACHT^{Nkx2.1-Cre-flox/flox} mice**

Previous experiments have highlighted the involvement of cholinergic tone in locomotion (Leanza et al. 1996; Mattsson et al. 2004; Martins-Silva et al. 2011; Martyn et al. 2012). Therefore, we assessed locomotion in VACHT^{Nkx2.1-Cre-flox/flox} mice using automated locomotor boxes. We found that VACHT^{Nkx2.1-Cre-flox/flox} mice were significantly hyperactive compared to controls (Two-way ANOVA shows an effect of time $F_{(11,154)}=15.07$, $p<0.0001$, an effect of genotype $F_{(1,14)}=12.75$, $p=0.0031$, and no interaction $F_{(11,154)}=1.293$, $p=0.233$, Figure 18A).

The regulation of motor activity by cholinergic signalling has been examined, however, the contribution of cholinergic input from distinct brain regions remains unclear. Previously, our group has reported that glutamate co-released from cholinergic striatal interneurons, but not acetylcholine, is involved in the regulation of locomotor activity in the striatum (Guzman et al. 2011). Because VACHT^{Nkx2.1-Cre-flox/flox} mice show decreased VACHT expression also in the striatum, we decided to further investigate the involvement of striatal vs. hippocampal/cortical cholinergic signalling. Our previous findings regarding the role of striatal cholinergic tone involved targeting of VACHT. Here we asked if striatal cholinergic tone is not involved in locomotion using mice with selective removal of ChAT in the striatum (ChAT^{D2-Cre-flox/flox}). In order to do this, we crossed the D2-Cre BAC transgenic mouse line generated by GENSAT with a
ChAT floxed mouse line (Misgeld et al. 2002). Previous work from our laboratory using this Cre mouse line demonstrated that Cre expression is restricted to the striatum (Guzman et al. 2011). Data analysis showed that ChAT$^{D2-Cre-flox/flox}$ mice were not hyperactive compared to ChAT$^{flox/flox}$ controls during the first hour of locomotor activity testing (Two-way ANOVA shows an effect of time $F_{(11,110)}=6.234$, $p<0.0001$, no effect of genotype $F_{(1,10)}=0.621$, $p=0.448$, and an interaction effect $F_{(11,110)}=2.077$, $p=0.0277$, Figure 18B). These findings suggest that striatal cholinergic tone does not seem to play a major role in modulating locomotor activity.
Figure 18. Locomotor activity in cholinergic-deficient mice. (A) Horizontal activity in an open field for VACHT$_{flox/flox}$ (white, n=8) and VACHT$_{Nkx2.1-Cre-flox/flox}$ (black, n=8) mice was measured over 1 hour period. (B) Horizontal activity in an open field for ChAT$_{flox/flox}$ (white, n=6) and ChAT$_{D2-Cre-flox/flox}$ (black, n=6) mice was measured over 1 hour period. (Data are mean ± SEM. *P<0.05, **P<0.01).
3.3.3 Metabolic analysis in VACHT-deficient mice

Cholinergic tone can regulate metabolic homeostasis (reviewed in (Picciotto et al. 2012)). To investigate whether decreased forebrain cholinergic tone affects homeostasis, VACHT<sup>NKx2.1-Cre-flox/flox</sup> mice were assessed in closed metabolic cages. Weight analysis of young adult mice (3 months old) indicated that forebrain VACHT-deficient mice are significantly leaner compared to age-matched controls ($t_{(16)}=5.398$, $p<0.0001$, Figure 19A). Metabolic analysis revealed no significant differences in respiratory exchange rate between VACHT<sup>NKx2.1-Cre-flox/flox</sup> in the light ($t_{(11)}=0.552$, $p=0.599$) or dark ($t_{(11)}=0.948$, $p=0.363$) cycles (Figure 19). Assessment of locomotor activity during the 24-hour period in the metabolic cages confirmed that VACHT-deficient mice were hyperactive in the dark cycle ($t_{(11)}=2.345$, $p=0.038$), but not the light cycle ($t_{(11)}=0.647$, $p=0.531$), compared to controls (Figure 19C).

Sleep analysis revealed that VACHT<sup>NKx2.1-Cre-flox/flox</sup> mice had significantly fewer sleep bouts cycles ($t_{(11)}=2.997$, $p=0.0121$, Figure 19D) and decreased sleep duration cycle ($t_{(11)}=2.357$, $p=0.033$, Figure 19E) during the dark cycle, but not the light cycle when compared to controls. Food consumption and water consumption showed similar profiles for both VACHT<sup>NKx2.1-Cre-flox/flox</sup> and VACHT<sup>flox/flox</sup> mice (Two-way RM ANOVA shows an effect of time $F_{(139,1529)}=457.4$, $p<0.0001$, no effect of genotype $F_{(1,11)}=1.618$, $p=0.250$, and an
interaction effect $F_{(139,1529)}=5.215$, $p<0.001$, Figure 19F) (Two-way RM ANOVA shows an effect of time $F_{(139,1529)}=647.2$, $p<0.0001$, no effect of genotype $F_{(1,11)}=5.076$, $p=0.065$, and an interaction effect $F_{(139,1529)}=15.27$, $p<0.0001$, Figure 19G). In addition, similar results were obtained for heat profile during 24-hour period in the metabolic cages (Two-way RM ANOVA shows an effect of time $F_{(139,1529)}=6.490$, $p<0.0001$, no effect of genotype $F_{(1,11)}=0.2990$, $p=0.594$, and no interaction $F_{(139,1529)}=1.125$, $p=0.161$, Figure 19H).
Figure 19. Metabolic analysis in VACHT<sup>Nkx2.1-Cre-flox/flox</sup> mice. (A) Body weight at time of metabolic cage analysis. (B) Respiratory exchange rate analysis. (C) Home cage locomotor activity. ON indicates when light is turned on, and OFF indicates when light is turned off in testing room. (D) Sleep bouts. (E) Sleep time. (F) Cumulative food consumption. (G) Cumulative water consumption. (H) Heat profile during 24-hour period in metabolic cages. VACHT<sup>Nkx2.1-Cre-flox/flox</sup> n=7, VACHT<sup>flox/flox</sup> n=6 (Data are mean ± SEM. *P<0.05, **P<0.01, ***P<0.0001).
3.3.5 Assessment of depressive-like behaviour in cholinergic-deficient mice

Previous experiments have shown that cholinergic signalling plays a critical role in modulating depressive-like behaviour in rodents (Caldarone et al. 2004). An increase in cholinergic tone leads to a pro-depressive phenotype, while blocking cholinergic signalling through either muscarinic, or nicotinic receptors, leads to anti-depressant-like effects (Rabenstein et al. 2006; Drevets et al. 2013). Furthermore, there is evidence suggesting that enhancing hippocampal cholinergic tone promotes depressive-like behaviours in mice (Mineur et al. 2013). Therefore, we tested whether deficient forebrain cholinergic tone has an effect on depressive-like behaviour. In the forced swim test, VACHTNkx2.1-Cre-flox/flox mice were similar to littermate controls in time spent immobile ($t_{(18)}=1.128$, $p=0.274$, Figure 20A), as well as the distance covered swimming ($t_{(18)}=0.539$, $p=0.595$, Figure 20B). We also assessed depressive-like behaviour in the tail-suspension test. Similar to the forced swim test, VACHTNkx2.1-Cre-flox/flox mice did not differ from littermate controls in the tail-suspension test ($t_{(9)}=1.305$, $p=0.224$, Figure 20C).

Next, in an attempt to dissect the specific contributions of striatal vs. hippocampal/cortical cholinergic signalling to depressive-like behaviours we tested ChATD2-Cre-flox/flox mice and controls on the same behavioural tests. We found that striatal-specific deficient mice displayed an anti-depressant-like
phenotype compared to controls in the immobility time in the forced swim test ($t_{10}=2.710$, $p=0.0219$, Figure 2A), and immobility time in the tail-suspension test ($t_{10}=2.301$, $p=0.044$, Figure 2C). These results indicate that decreased striatal cholinergic signalling leads to an anti-depressive like phenotype in mice, suggesting a role for striatal cholinergic signalling in the modulation of depression.
Figure 20. Depressive-like analysis in forebrain cholinergic-deficient mice.
(A) Immobility time in the final four minutes of testing in the forced swim test. (B) Total distance swam in the final four minutes of testing in the forced swim test, $\text{VACHT}^{\text{Nkx2.1-Cre-flox/flox}}$ n=10, $\text{VACHT}^{\text{flox/flox}}$ n=10. (C) The total immobility time in the final four minutes of testing in the tail-suspension test $\text{VACHT}^{\text{Nkx2.1-Cre-flox/flox}}$ n=5 $\text{VACHT}^{\text{flox/flox}}$ n=6. (Data are mean ± SEM).
Figure 21. Depressive-like analysis in striatal cholinergic-deficient mice. (A) Immobility time in the final four minutes of testing in the forced swim test. (B) Total distance swam in the final four minutes of testing in the forced swim test, ChAT\textsuperscript{D2-Cre-flox/flox} n=6, ChAT\textsuperscript{flox/flox} n=6. (C) The total immobility time in the final four minutes of testing in the tail-suspension test ChAT\textsuperscript{D2-Cre-flox/flox} n=6, ChAT\textsuperscript{flox/flox} n=6. (Data are mean ± SEM).
3.4 Determining the contributions of forebrain cholinergic signalling to cognitive functioning

3.4.1 *Forebrain cholinergic signalling is required for paired associates learning*

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). The PAL task requires sophisticated processing of information for proper association of images with specific locations. VACHT\textsuperscript{Nkx2.1-Cre-flox/flox} mice and their matched controls were assessed on the PAL task using an automated touchscreen system (Figure 22A).

Prior to being subjected to the PAL task, both experimental groups go through a number of different types of training sessions (initial touch, must touch stimuli, must initiate and punish incorrect) to learn how to operate the touchscreen. At these sessions they learn for instance, to touch the screen when a stimulus is presented and to initiate the task by inserting the head into the reward chamber. This pre-training also includes the “punish incorrect” phase, when only one stimulus is presented randomly in one of the 3 screens, and mice are taught to touch the screen that shows the stimulus. Mice from both experimental groups were able to reach criterion in all these phases of the pre-training. No differences
were observed between the two genotypes ($t_{(12)}=0.0749$, Figure 22B). These data also indicate that VAChT-deficient mice are able to learn that they need to touch the screen when an image is shown, which argues that VAChT-deficient mice do not present any major visual impairment.

During the course of the 9 weeks following training, mice were tested on the dPAL task. We observed that control mice significantly improved their accuracy performance, while VACHT$^{Nkx2.1-Cre-flox/flox}$ did not (Two-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 $F_{(8,48)}=2.871$, $p=0.0074$, Figure 22C). VACHT$^{floxflox}$ mice (controls) were able to improve performance reaching 77% ± 1 accuracy by week 9 (Figure 22C). In contrast, peak accuracy performance of VACHT$^{Nkx2.1-Cre-flox/flox}$ mice in the dPAL task during the same period was 58% ± 2 (Figure 22C). Although VACHT$^{Nkx2.1-Cre-flox/flox}$ mice were able to perform the 36 trials required in each one-hour session, they failed to associate the stimulus to its correct location. Their poor performance was also reflected in the number of correction errors performed (Figure 22D). VACHT$^{Nkx2.1-Cre-flox/flox}$ 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 (Two-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 $F_{(8,48)}=1.224$, $p=0.0306$, Figure 22D). Correct response latency was not different between the two groups over
the course of 9 weeks (Two-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 $F_{(8,48)}=1.195$, $p=0.3220$, Figure 22E). Furthermore, VACht^{Nkx2.1-Cre^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 poor performance (Two-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 $F_{(8,48)}=0.6061$, $p=0.7681$ Figure 22F). In summary, VACht^{Nkx2.1-Cre^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. Videos of mice performing in the PAL task can be downloaded from:

http://cercor.oxfordjournals.org/content/early/2016/01/22/cercor.bhv349.long
Figure 22. VACH\textsuperscript{T^NKx2.1-Cre-flox/flox} mice display impairments in the acquisition of PAL. (A) Image depicting a mouse performing the task, where the flower is shown as S+ and the airplane as S-. (B) Number of sessions required by both experimental groups to reach criterion during the operant conditioning, pretraining, and training phases (Habituation; phase 1) (Initial touch training; phase 2) (Must touch stimuli training; phase 3) (Must initiate; phase 4)(Punish incorrect; phase 5). (C-F) Data for acquisition of the dPAL task for VACH\textsuperscript{T^flox/flox} (n=7 clear squares) and VACH\textsuperscript{T^NKx2.1-Cre-flox/flox} (n=7 dark circles) mice. Each week represents five testing sessions of 36 trials each (C) Mean accuracy. (D) Mean correction errors. (E) Response latency. (F) Reward collection latency. (Data are mean ± SEM. *P<0.05, **P<0.01, ***P<0.0001).
3.4.2 VACH\textsuperscript{T}\textsuperscript{Nkx2.1-Cre-flox/flox} mice exhibit a mild impairment in spatial memory

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). Here we evaluated spatial memory in VACH\textsuperscript{T}\textsuperscript{Nkx2.1-Cre-flox/flox} mice. Our data showed that spatial learning on the MWM was mildly affected in VACH\textsuperscript{T}\textsuperscript{Nkx2.1-Cre-flox/flox} mice (3-6 month old) when compared to age-matched controls (Two-way RM ANOVA shows a significant effect of days $F_{(3,30)}=37.2$, $p<0.0001$, no effect of genotype $F_{(1,10)}=4.790$, $p=0.0535$, and no interaction $F_{(3,30)}=0.9567$, $p=0.4259$, Figure 23A). Both groups improved in the distance taken to reach the target quadrant during the course of 4 days, however VACH\textsuperscript{T}\textsuperscript{Nkx2.1-Cre-flox/flox} mice were less effective to find the platform (Two-way RM ANOVA shows a significant effect of days $F_{(3,30)}=32.2$, $p<0.0001$, an effect of genotype $F_{(1,10)}=12.58$, $p=0.0053$, and no effect of interaction $F_{(3,30)}=0.5597$, $p=0.6458$, Figure 23B). No significant differences were observed between the two groups in the speed to reach the platform (Two-way RM ANOVA shows a significant effect of days $F_{(3,30)}=14.11$, $p<0.0001$, no effect of genotype $F_{(1,10)}=0.9260$, $p=0.3586$, and no interaction $F_{(3,30)}=1.805$, $p=0.1675$, Figure 23C). On the probe trial of the MWM, both groups of mice spent significantly more time in the target quadrant compared to the
opposite quadrant (Two-way ANOVA shows a significant effect of quadrant, $F_{(3,80)}=39.58$, $p<0.0001$, and an interaction effect $F_{(3,80)}=2.914$, $p=0.0394$, Figure 23D), post-hoc analysis revealed that both groups spent significantly more time in the target quadrant. However, $\text{VAChT}^{Nkx2.1-\text{Cre-flox/flox}}$ mice had significantly fewer platform crosses compared to littermate controls ($t_{(20)}=2.795$, $p=0.0112$, Figure 23E), suggesting that they exhibit a mild impairment in spatial memory recall compared to controls.

We also analyzed the strategies (Figure 24A) these mice used to learn where the platform is located. As observed in Figure 24B, both $\text{VAChT}^{Nkx2.1-\text{Cre-flox/flox}}$ and $\text{VAChT}^{\text{flox/flox}}$ mice used a more direct strategy to reach the platform (strategies 5/6/7) and there was no difference between genotypes, indicating no major impairments in learning in $\text{VAChT}^{Nkx2.1-\text{Cre-flox/flox}}$ mice. To assess cue-driven learning in $\text{VAChT}^{Nkx2.1-\text{Cre-flox/flox}}$ mice, the cued version of the MWM was used. Our data showed that cued learning on the MWM was not affected in $\text{VAChT}^{Nkx2.1-\text{Cre-flox/flox}}$ mice (3-6 month old) when compared to age-matched controls (Two-way RM ANOVA shows a significant effect of days $F_{(1,26)}=15.03$, $p=0.0006$, no effect of genotype $F_{(1,26)}=0.1468$, $p=0.7048$, and no interaction $F_{(1,26)}=0.0771$, $p=0.7834$, Figure 25A). Furthermore, no significant differences were observed between the two groups in the speed to reach the platform (Two-way RM ANOVA shows a significant effect of days $F_{(1,26)}=11.78$, $p=0.0020$, no effect of genotype $F_{(1,26)}=0.07437$, $p=0.7872$, and no interaction $F_{(1,26)}=0.01525$, $p=0.9027$, Figure 25B)
Figure 23. Performance of VACHT\textsuperscript{Nkx2.1-Cre-flox/flox} mice in the MWM. VACHT\textsuperscript{flox/flox} (clear squares, n=11) and VACHT\textsuperscript{Nkx2.1-Cre-flox/flox} (dark circles, n=11) mice were tested in the spatial paradigm of the MWM. The data average four 90-s trials per day were plotted. (a) Latency, (b) Distance, (c) Speed, (d) 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. (e) Number of platform crosses during the probe trial. (f) Representative path traces for two VACHT\textsuperscript{flox/flox} and two VACHT\textsuperscript{Nkx2.1-Cre-flox/flox} mice in the probe trial. The target quadrant is in the lower right. Data are mean ± SEM. *P<0.05, **P<0.01. T, target; O, opposite; L, left; R, right.
Figure 24. Learning strategies of VACHT\textsuperscript{Nkx2.1-Cre-flox/flox} mice. (A) Strategy plot reflecting the mean strategy-recruitment values for the first and fourth trials of each day for young mice. (B) 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)
Figure 25. Cued memory analysis of VACHT<sup>Nkx2.1-Cre-flox/flox</sup> mice. Mice were subject to the cued version of the MWM. A. Primary latency to find the platform. B. Speed traveled to the platform. The average of four 90-s trials per day is plotted. *p<0.05, VACHT<sup>flox/flox</sup> n=6, VACHT<sup>Nkx2.1-Cre-flox/flox</sup> n=8.
3.4.3 \( VACht^{Nkx2.1-Cre-flox/flox} \) mice show deficits in cognitive flexibility

Previous work has shown that rats with 192 IgG-saporin lesions restricted to NBM show cognitive flexibility impairments (Cabrera et al. 2006). To investigate cognitive flexibility, \( VACht^{Nkx2.1-Cre-flox/flox} \) mice were tested on the reversal learning protocol of the MWM. During the course of 4 days, while control mice significantly improved in their latency to find the hidden platform \( VACht^{Nkx2.1-Cre-flox/flox} \) mice showed a significant deficit (Two-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 no interaction \( F_{(3,30)}=1.501, p=0.2342 \), Figure 2A-C). Notably, on the probe trial, control mice spent considerably more time in the target quadrant compared to the other quadrants (Two-way ANOVA shows a significant effect of quadrant, \( F_{(3,80)}=7.226, p=0.0002 \), and an interaction effect \( F_{(3,80)}= 3.133, p=0.0301 \), Figure 2D), while \( VACht^{Nkx2.1-Cre-flox/flox} \) mice visited all quadrants almost equally. The number of platform crosses was also higher for control mice compared to VACht deficient mice (\( t_{(20)}=2.797, p=0.0111 \), Figure 2E). These results indicate that, different from control mice, \( VACht^{Nkx2.1-Cre-flox/flox} \) mice were unable to extinguish the previously learned position and relearn the new position of the hidden platform.
Figure 26. Reversal learning is affected in \( \text{VACHT}^{\text{Nkx2.1-Cre-flox/flox}} \) mice. \( \text{VACHT}^{\text{flox/flox}} \) (clear squares, \( n=11 \)), \( \text{VACHT}^{\text{Nkx2.1-Cre-flox/flox}} \) (dark circles, \( n=11 \)) were tested in the reversal paradigm of the MWM. Data averages 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 two \( \text{VACHT}^{\text{flox/flox}} \) and two \( \text{VACHT}^{\text{Nkx2.1-Cre-flox/flox}} \) in the probe trial. The target quadrant is in the upper left. Data are mean ± SEM. \( *P<0.05, **P<0.01 \). T, target; O, opposite; L, left; R, right.
To investigate the possibility of Cre-recombinase mediating these spatial impairments in VACHT-mutant mice, we assessed spatial navigation using the MWM task in mice expressing Cre-recombinase (Nkx2.1-Cre). As observed in Figure 27A latency to reach the platform was not different between groups (Two-way RM ANOVA shows an effect of days $F_{(3,12)}=90.03$, $p<0.0001$, no effect of genotype $F_{(1,4)}=0.020$, $p=0.894$, and no interaction $F_{(3,12)}=1.102$, $p=0.3861$). Similar results were obtained for distance travelled (Two-way RM ANOVA shows an effect of days $F_{(3,12)}=52.81$, $p<0.0001$, no effect of genotype $F_{(1,4)}=0.182$, $p=0.691$, and no interaction $F_{(3,12)}=0.806$, $p=0.514$, Figure 27B). Furthermore, no differences were observed in speed (Two-way RM ANOVA shows an effect of days $F_{(3,12)}=5.927$, $p<0.0001$, no effect of genotype $F_{(1,4)}=0.052$, $p=0.830$, and no interaction $F_{(3,12)}=0.556$, $p=0.653$, Figure 27C). In the probe trial, Nkx2.1-Cre mice did not differ from C57BL/6 littermate controls in terms of preference for the target quadrant (Two-way ANOVA shows a significant effect of quadrant, $F_{(3,24)}=46.63$, $p<0.0001$, no effect of genotype, $F_{(1,8)}=0.167$, and no interaction $F_{(3,24)}=0.990$, $p=0.414$, Figure 27D). To assess cognitive flexibility, Nkx2.1-Cre mice were tested on the reversal learning protocol of the MWM. During the course of 4 days, both C57BL/6 and Nkx2.1-Cre mice significantly improved in their latency to find the hidden platform (Two-way RM ANOVA shows a significant effect of days $F_{(3,12)}=8.809$, $p=0.0023$, no effect of genotype $F_{(1,4)}=0.368$, $p=0.576$, and no interaction $F_{(3,12)}=0.347$, $p=0.792$, Figure 27E). Similarly, no differences were observed in distance (Two-way RM ANOVA shows a significant effect of days $F_{(3,12)}=12.92$, $p=0.0005$, no effect of genotype $F_{(1,4)}=0.3861$).
0.784, p=0.425, and no interaction $F_{(3,12)}=0.937$, p=0.452, Figure 27F) or speed (Two-way RM ANOVA shows a significant effect of days $F_{(3,12)}=5.423$, p=0.013, no effect of genotype $F_{(1,4)}=0.248$, p=0.644, and no interaction $F_{(3,12)}=0.484$, p=0.699, Figure 27G). Notably, on the probe trial, control and Nkx2.1-Cre mice spent considerably more time in the target quadrant compared to the other quadrants (Two-way ANOVA shows a significant effect of quadrant, $F_{(3,24)}=13.5$, p<0.0001, no effect of genotype, $F_{(1,8)}=, p=0.328$, and no interaction $F_{(3,24)}=0.271$, p=0.845, Figure 27H). Together, these results indicate that Cre expression has no profound consequences on neuromuscular performance, spatial acquisition, and cognitive flexibility.
Figure 27. Performance of Nkx2.1-Cre mice in the MWM. WT littermate controls (C57BL/6, clear squares, n=6) and Nkx2.1-Cre (dark circles, n=6) mice were tested in the spatial paradigm of the MWM. The data average four 90-s trials per day were plotted. (A) Latency, (B) distance, (C) speed. (D) Probe trial data indicating 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. Reversal learning data in the MWM. (E) Latency, (F) distance, (G) speed. (H) Probe trial data indicating 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. (Data are mean ± SEM. *P<0.05, **P<0.01, ***P<0.0001).
3.4.4 \textit{VACHT}^{Nkx2.1-Cre-flox/flox} mice show impaired performance in a two-choice location discrimination task

Because young (3-6 month old) VACHT deficient mice presented impairments in neurogenesis, we sought to investigate whether these deficits have consequences on performance on a task thought to be dependent on adult hippocampal neurogenesis (Clelland et al. 2009). \textit{VACHT}^{Nkx2.1-Cre-flox/flox} mice were tested on a two-choice spatial discrimination task. Mice were required to touch one of two illuminated screens (e.g. S+ is in the most left screen) until a criterion of 9/10 consecutive correct trials were achieved. Once criterion was reached, a reversal occurred where the other location (e.g. S+ is now in the rightmost screen). In addition, the separation of the illuminated screens was presented with either a high degree of separation (easy; 3 blank screens separating the illuminated screens), or a low degree of separation (hard; 1 blank screen separating the illuminated screens) (Figure 28A). VACHT-deficient mice performed similarly to controls in the number of trials they took to reach criterion in the high separation ($t_{(6)}=0.5541$, $p=0.6034$) (Figure 28B). However, at the low separation, \textit{VACHT}^{Nkx2.1-Cre-flox/flox} mice exhibited impaired performance in the task, highlighted by an increased number of trials to reach criterion compared to controls ($t_{(6)}=5.137$, $p=0.0119$) (Figure 28B).
To evaluate the number of newly generated neurons during performance on the acquisition of the two-choice location discrimination task, mice were injected with BrdU on the day they began the acquisition. After completion of behavioural testing, mice were killed and their brains were analyzed for BrdU expression. Immunoreactivity data showed a significant decrease in the number of newborn cells in the subgranular zone of this hippocampus in VACHT^{Nkx2.1-Cre-flox/flox} mice compared to controls ($t_{(4)}=3.366$, $p=0.0282$) (Figure 28C-D). To note, it does not appear that performance in the two-choice location discrimination task significantly enhanced BrdU labelling in both controls and VACHT-deficient mice, compared to baseline expression. Together, our findings suggest that hippocampal cholinergic tone not only disrupts neurogenesis, but also has a functional consequence on pattern separation.
Figure 28. Elimination of hippocampal cholinergic signalling disrupts pattern separation. (A) Schematic of stimuli presented in either the high (left) or low (right) separations (B) The number of trials to reach criterion in the high and low separations in the two-choice spatial discrimination task for VACHT^{Nkx2.1-Cre-flox/flox} and controls (C-D) BrdU expression and quantification in the dentate gyrus of controls and VACHT^{Nkx2.1-Cre-flox/flox} mice. (n=3, data are mean ± SEM. *P<0.05).
3.4.5 *Long-lasting cholinergic depletion impairs learning strategies*

Given that VACHT<sup>Nkx2.1-Cre-flox/flox</sup> mice presented age-dependent pathology in the hippocampus, we then asked whether long-lasting cholinergic failure worsens cognitive function. To answer this question we chose to investigate aged mice in the spatial version of the MWM, as young (3-6 months old) VACHT-deficient mice showed only minor deficits in this task (Figure 24). As can be observed in (Figure 29A-B), aged (11-14 month old) 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 of the MWM task.

On the probe trial, VACHT-deficient mice showed no preference to the target quadrant, whereas controls did (Figure 29C). Furthermore, aged VACHT-deficient mice used distinct strategy preferences to find the platform, indicating that their deteriorated performance was due to modified learning capacities. The search strategies used by each animal was based on a fixed set of criteria (Figure 29D). While at a young age, both controls and VACHT<sup>Nkx2.1-Cre-flox/flox</sup> mice predominantly used more direct strategies to reach the platform (strategies 5/6/7, Figure 24); aged VACHT-deficient mice used random swimming predominantly as their strategy to acquire the task (strategy 2, Figure 29E). On the other hand, aged control mice maintained the use of more direct strategies. Aged VACHT-deficient
mice also exhibited deficits in the probe trial (Figure 29E). Taken together, these results suggest that long-term cholinergic deficiency in VACht\textsuperscript{Nkx2.1-Cre-flox/flox} mice worsened spatial information acquisition.
Figure 29. Performance of aged VACH\textsuperscript{T}\Nkx2.1-Cre-flox/flox mice in the MWM. VACH\textsuperscript{T}\flox/flox (grey circles, n=8) and VACH\textsuperscript{T}\Nkx2.1-Cre-flox/flox (red squares, n=8) mice were tested in the spatial paradigm of the MWM. The data average four 90-s trials per day were plotted. (A) Latency, (B) Distance, (C) 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. (D) Representative examples of the 7 classified criteria to score the strategies mice used to perform in the MWM. Strategies are color coded. (E) Strategy plot reflecting the mean strategy-recruitment values for the first and fourth trials of each day for aged 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 VACH-T-deficient mice. (n= 8, data are mean ± SEM. *P<0.05 **P<0.01).
Chapter 4

4. Discussion

4.1 Generation of a forebrain-specific cholinergic deficient mouse model

Although cholinergic innervations are widespread in the CNS and ACh is suggested to be involved in several cognitive and behavioural functions, the exact role of ACh in these processes is not well understood. Likewise, information about the specific cholinergic nuclei involved in the modulation of these brain functions is still incomplete. One of the difficulties in understanding the exact role of ACh in behavioural processes was the lack of a good animal model. Previous work used immunotoxins to mimic cholinergic dysfunction in mice and rats (Berger-Sweeney et al. 1994; Parent and Baxter 2004). Ablation of cholinergic neurons using immunotoxins provided inconsistencies regarding the involvement of the basal forebrain cholinergic system in memory tasks and it is particularly problematic in mice, as immunotoxins are not as efficient (Baxter and Gallagher 1996; Walsh et al. 1996; Janis et al. 1998). Immunotoxin ablations display poor specificity depending on the dose used (Moreau et al. 2008) and several side effects have been reported in mice, including seizures (Berger-
Sweeney et al. 2001). Additionally, differences in size and location of the lesions; as well as differences in the extent of cholinergic neuron ablation on individual animal difficult analysis of the results. Furthermore, because cholinergic neurons from different brain regions have been shown to co-release ACh with other classical neurotransmitters toxin-based approaches do not allow differentiation of the role of ACh from the other neurotransmitter co-secreted, such as glutamate or GABA, by these cholinergic neurons (Guzman et al. 2011; Higley et al. 2011; Saunders et al. 2015). Genetic targeting using the Cre/lox system has provided a means to investigate how compromised forebrain release of ACh influences cognitive performance, and molecular hallmarks of AD.

In this thesis we used the Cre-loxP system to specifically delete cholinergic genes from the forebrain of mice and mimic cholinergic dysfunction. Specifically, we used the Nkx2.1 promoter to restrict Cre expression to the forebrain. Biochemical analyses revealed that VACHT expression was severely compromised in the forebrain areas, including the hippocampus. Previously, work from our laboratory used the Six3-Cre promoter to target VACHT expression (VACHT\textsuperscript{Six3-Cre-flox/flox}) in the mouse forebrain (Martyn et al. 2012). These mice are very similar to the VACHT\textsuperscript{Nkx2.1-Cre-flox/flox} mice in that they show specificity to forebrain regions and present similar behavioural phenotypes. When generating VACHT\textsuperscript{Six3-Cre-flox/flox} mice however, there was a propensity for germ line deletion in the VACHT gene in these animals, resulting in the generation of hemizygous del allele that would be present in all cells of the animal. These mice show 50%
decrease in VACht expression in all cholinergic expressing cells, including all brain regions and the peripheral nervous system. Our current mouse model circumvents these concerns as Nkx2.1-Cre mice are generated using a BAC clone. Because BAC clones are usually very long sequences, they generally contain all the regulatory regions necessary for cell specific gene expression and therefore, mice generated with BAC clones have a reduced chance of ectopic Cre expression. Furthermore, we show that Cre expression per se does not interfere with cognitive nor motor behaviours. Thus, our data suggest that VACht^{Nkx2.1-Cre-flox/flox} is a good model to investigate how decreased ACh released from forebrain cholinergic neurons influence brain function both at cellular and behavioural level.

4.2 Long-term cholinergic loss and Alzheimer’s-like pathology

Neurodegenerative changes in the basal forebrain cholinergic system are characteristic in AD (Teipel et al. 2014) and prodromal AD (Teipel et al. 2016). Long-term administration of drugs with anticholinergic side effects increases an individual’s risk of developing AD (Gray et al. 2015). Moreover, use of drugs with medium or high anticholinergic activity correlates with poorer cognitive performance and increased brain atrophy in older adults (Risacher et al. 2016). Our data show that aged VACht-deficient mice display molecular hallmarks that are common to AD and neurodegenerative diseases. We observed that aged
VACHT-deficient mice presented alterations in tau and an age-dependent increase in protein aggregation. This strongly supports a relationship between cholinergic failure and pathology in AD. Aging has been linked with increased neuronal vulnerability (Mattson and Magnus 2006). Moreover, studies in rats have demonstrated that basal forebrain cholinergic neurons are particularly vulnerable to aging (Biegon et al. 1986; Altavista et al. 1990; Casu et al. 2002). It has been proposed that the selective vulnerability of the basal forebrain cholinergic system pertains to the higher demand in these cholinergic neurons for glucose production (Szutowicz et al. 2006). Indeed, gene expression analysis in rats displayed enhanced metabolic activity in basal forebrain cholinergic neurons, in comparison to brain stem cholinergic neurons (Baskerville et al. 2008). Our findings are in line with the notion that basal forebrain cholinergic dysfunction leads to increased neuronal vulnerability (Altavista et al. 1990; Casu et al. 2002), but we also add that age-related cholinergic deprivation may have a consequence on target cells’ health, and worsens cognitive performance.

The age-dependent increase of intracellular Thioflavin-S staining observed in the hippocampus of VACT\textsuperscript{Nkx2.1-Cre-flox/flox} mice suggests age-dependent protein aggregation. Thioflavin-S is a fluorescent dye used to detect the aggregates of $\beta$-sheet proteins in cells (Bulic et al. 2007; Lim et al. 2014). While the identity of these aggregates remain unclear, it is worth noting that we observed tau hyperphosphorylation at Thr231 in VACHT-deficient mice. Because NFTs occur as intracellular deposits (Iqbal and Grundke-Iqbal 2006) and contain $\beta$-sheets
when aggregated (Maeda et al. 2007), it is possible that these aggregates may be composed of tau. The pathophysiology of protein aggregation in neurodegenerative diseases including AD has been hypothesized to occur due to a pathogenic propagation and/or selective vulnerability of groups of neurons. According to the 'pathogenic spread' hypothesis (Brettschneider et al. 2015; Walsh and Selkoe 2016), aggregation of proteins occur in one brain region and propagate from neuron to neuron and thus spread into connected brain regions. Whether this propagation occurs in our mice remains to be established. On the other hand, the 'selective vulnerability' hypothesis suggests that in response to certain stressful conditions, such as aging, protein aggregation is triggered in neurons that are particularly vulnerable (Walsh and Selkoe 2016). Protein aggregates appear first in the cells most susceptible to stressful conditions and with time aggregates emerge in less-susceptible cells (Mattson and Magnus 2006). Based on this hypothesis, our findings suggest that impaired cholinergic neurotransmission significantly increases stress and vulnerability of hippocampal cells. This hypothesis also suggests that protein aggregation is mediated by the spread of diffusible metabolic factors that result in the propagation of these stressful conditions to an adjacent neuron, as opposed to direct physical transfer of protein aggregates (Walsh and Selkoe 2016). Furthermore, these possibilities may not be mutually exclusive.

Our data demonstrated that aged VAChT-mutants show increased T231/S235 phosphorylation, but not at S262. There have been 25 abnormal sites described
at which tau in PHFs is hyperphosphorylated (Hanger et al. 1998). Hyperphosphorylation of tau can increase neuronal vulnerability via destabilization of microtubules and disruption of axonal transport, which ultimately leads to neuronal death (Billingsley and Kincaid 1997; Iqbal et al. 1998; Brion et al. 2001). The exact relationship between cholinergic signalling and tau pathology has not been established. However, human studies show that intraneuronal tau pathology occurs prior to basal forebrain cholinergic neuronal loss, which inversely correlated with tests of cognitive function (Vana et al. 2011). Furthermore, cholinergic dysfunction occurs early in MCI and early AD, even prior to cholinergic neuronal loss (Auld et al. 2002; Picciotto and Zoli 2002; Mufson et al. 2007). Our data demonstrated that aged VACHT-mutants show increased T231/S235 phosphorylation, but not at S262. There have been 25 abnormal sites described at which tau in PHFs is hyperphosphorylated (Hanger et al. 1998). Hyperphosphorylation of tau can increase neuronal vulnerability via destabilization of microtubules and disruption of axonal transport, which ultimately leads to neuronal death (Billingsley and Kincaid 1997; Iqbal et al. 1998; Brion et al. 2001). The exact relationship between cholinergic signalling and tau pathology has not been established. It is interesting to note that in human studies it has been shown that intraneuronal tau pathology occurs prior to basal forebrain cholinergic neuronal loss, and is inversely correlated with cognitive dysfunction (Vana et al. 2011). Tau hyperphosphorylation at Thr231 is consistently reported in AD brains (Jicha et al. 1997; Vincent et al. 1997), suggesting that the formation of PHFs in AD could be highly dependent on tau phosphorylation on Thr231. Our
data further support a role for hyperphosphorylation of tau at Thr231 contributing to inhibition of binding of tau to microtubules (Sengupta et al. 2006).

Phosphorylation of tau is mediated by several protein kinases, including GSK-3, which co-localizes with neurofibrillary tangles in AD (Pei et al. 1998; Pei et al. 1999). In addition, mice overexpressing GSK-3 in the forebrain present tau hyperphosphorylation and apoptosis, which could be rescued with silencing of GSK-3 activity. Furthermore, GSK3β-mediated hyperphosphorylation of tau leads to an array of impairments, including disruption of LTP (Hooper et al. 2007) and in vitro cell death (Zheng et al. 2002). Interestingly, genome-wide transcriptome analysis of the hippocampus of VACHT-deficient mice suggests that the PI3-AKT pathway is upregulated compared to control mice. Western blot analysis revealed that its downstream target GSK3 activity was increased (Kolinsyk et al. 2016, submitted). Because tau phosphorylation can occur due to the activity of GSK3β, which subsequently leads to impairments in synaptic plasticity (Hooper et al. 2007), it could serve as a potential target. Importantly, chronic treatment of aged VACHTNkx2.1-Cre-flox/flox mice with the GSK3 inhibitor AR-A014418, known to significantly reduce neuritic plaque formation and rescued memory impairments in AD transgenic mice (Ly et al. 2013), led to a significant decrease in the levels of tau Thr231 hyperphosphorylation. Interestingly however, treatment of AR-A014418 in VACHTNkx2.1-Cre-flox/flox mice was neither able to reverse protein aggregation by Thioflavin S, nor caspase-3 activation, compared to saline treated VACHTNkx2.1-Cre-flox/flox mice (Kolinsyk et al. 2016, under review).
Given that treatment with AR-A014418 showed promise in terms of rescuing tau hyperphosphorylation in VACHT-deficient mice, future experiments should involve genetic targeting of GSK3 genes in VACHT<sup>Nkx2.1-Cre<sup>−/−</sup>flox/flox</sup> mice to test the long-term outcomes of inhibition of GSK3 activity to AD-related pathology and cognitive impairments.

Experiments have demonstrated that immunolesioning the basal forebrain cholinergic neurons in 3xTg-AD mice significantly increased neuropathology, including tau hyperphosphorylation (Hartig et al. 2014). In 3xTg-AD mice, which present amyloidosis and tau pathology, administration of a selective M1-agonist was able to attenuate tau pathology and amyloid pathology (Caccamo et al. 2006). Similarly, genetic deletion of M1 mAChRs in 3xTg-AD mice exacerbated tau hyperphosphorylation and amyloid pathology (Medeiros et al. 2011). These findings suggest that M1 mAChR activity may mediate tau hyperphosphorylation along with amyloidosis. However, it should be noted that experiments reported in Alzheimer’s mouse models are usually a result of a change in pathology that already exists. This is very different from our results in which cholinergic failure per se can trigger these pathological hallmarks similar to AD. Whether hyperphosphorylation of tau seen in our VACHT-deficient mice occurs due to aberrant M1 mAChR activity remains to be elucidated.

Our studies also reveal that aged-VACHT deficient mice presented MC1-positive immunoreactivity in the hippocampus, suggesting a conformational change of tau...
in these mice. The MC1 antibody detects the extreme N terminus and the third binding domain repeat of tau, which is dependent on the folding of tau (Jicha et al. 1997). Interestingly, experiments have demonstrated that this misfolding occurs in close proximity of Thr231, suggesting that hyperphosphorylation of tau on Thr231 may trigger conformational changes as observed in VACHT mutant mice (Jicha et al. 1997). 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). Hyperphosphorylation of tau and the associated age-dependent increase in protein aggregation in VACHT mutant mice suggest that decreased cholinergic tone elicits tauopathy.

Our results also showed that selective cholinergic inhibition to the hippocampus in aged control mice significantly increased immunoreactivity of activated caspase-3, a marker of apoptosis, in AAV8-Cre injected mice compared to GFP-injected controls. On the other hand, AAV8-Cre injected mice did not differ from controls in the number of silver deposits and tau Thr231 hyperphosphorylation. It is possible that these differences result from the fact that the time and the extent of cholinergic loss may have been insufficient to trigger these alterations in AAV8-Cre mice. It is also possible that developmental loss of cholinergic tone had a role on triggering tau pathology however, the fact that protein aggregation and tau hyperphosphorylation was not observed in young VACHT-mutants
argues against a prominent role for developmental ACh on tau pathology. Future studies using chemogenetics to specifically silence basal forebrain cholinergic neurons may help us to further clarify the effect of long-term cholinergic loss in AD-like pathology.

In line with an age dependent neuronal vulnerability in the hippocampus, we found that aged, but not young, VAChtT-deficient mice were severely impaired in their performance in the MWM task compared to age-matched controls. Using detailed analysis of precision strategies during training, we found that at a young age both cholinergic-deficient mice and controls were able to utilize more precise spatial strategies, and showed intact retention in the probe trial. Similarly, young adult mice lacking the β2 nAChR subunit are normal in the MWM; when aged however, these mice displayed robust impairments in the MWM (Zoli et al. 1999). To note, mice lacking the β2 nAChR subunit present progressive cell death in the hippocampus and cortex (Zoli et al. 1999), which could influence spatial navigation. Indeed, poor performance and acquisition on the MWM task has been associated with loss of neurons in the hippocampus (Olsen et al. 1994). In line with these studies, data from our laboratory show that long-term cholinergic loss decreased the number of neurons in the hippocampus (Kolisnyk et al. 2016, under review). These findings may be a major factor underlying the deterioration in cognitive performance in VAChtT-deficient mice.
4.2.1 Neuroinflammation

In this thesis we demonstrated that inflammatory cytokine levels as well as microglial activation are up regulated in the hippocampi of VACHT-deficient mice. Central neuroinflammation has been shown to be a key mechanism in the pathogenesis of AD (Wyss-Coray 2006; Heneka et al. 2014). The identification of associations between mutations in genes such as the triggering receptor expressed on myeloid cells 2 (TREM2) and the development of AD suggest a critical role of the innate inflammatory response in the pathogenesis of AD (Guerreiro et al. 2013; Jonsson et al. 2013). Microglial activation is necessary for remodelling synapses, synaptic plasticity, and for responding to alterations in neuronal health (Prokop et al. 2013; Prinz and Priller 2014). In vitro, soluble Aβ oligomers bind to various microglial receptors, which results in the production of inflammatory cytokines. Up regulation of inflammatory cytokines such as IL-1, IL-6, and tumour necrosis factor (TNF) has been observed in mouse models of AD (Benzing et al. 1999; Abbas et al. 2002). Another function of microglia is the clearing of Aβ through receptor-mediated phagocytosis (Prokop et al. 2013; Heppner et al. 2015). Recent findings demonstrate that activating microglia in APP/PS1 mice by IL-33 injections enhances Aβ phagocytic activity and decreased central proinflammatory gene expression (Fu et al. 2016). In the same study, cognitive impairments in APP/PS1 mice were reversed, suggesting a critical role for microglial for Aβ-related pathology and cognitive impairments in AD (Fu et al. 2016). Alternatively, Aβ can impair microglial function as APP/PS1
AD mice display marked reductions in levels of Aβ-binding scavenger receptor and the Aβ-degrading enzyme (Hickman et al. 2008). Furthermore, abnormalities in microglial function have been shown to significantly attenuate levels of trophic factors, including brain-derived neurotrophic factor (BDNF) (Parkhurst et al. 2013). Indeed, BDNF produced by microglia plays a role in motor learning, and increases neuronal tropomyosin-related kinase receptor B phosphorylation, a key modulator of synaptic plasticity (Parkhurst et al. 2013). Whether this increase in microglial activation due to long-term cholinergic loss plays a role in deteriorating cognition and in triggering age-related pathology warrants further investigation.

Cholinergic neurons in the basal forebrain appear to be selectively vulnerable to systemic administration of endotoxins, such as LPS (Wenk and Willard 1998; Willard et al. 2000). Systemic injections of LPS in mice with selective lesions of basal forebrain cholinergic neurons significantly impaired working memory in lesioned mice, without having an effect in non-lesioned mice (Field et al. 2012). These findings suggest that decreased basal forebrain cholinergic tone accentuates inflammatory responses to LPS and impair cognitive performance. Further evidence suggests that donepezil inhibits lipopolysaccharide-mediated increase in proinflammatory cytokines in the rat brain (Tyagi et al. 2010). Administration of methyllycaconitine, a α7 nAChR antagonist blocked the protective effect, suggesting that the protective role of donepezil in neuroinflammation is at least in part mediated via α7 nAChRs (Tyagi et al. 2010). Moreover, there is evidence-linking neuroinflammation to nicotinic receptor
activity as aged mice lacking the β2 subunit exhibit high levels of astro- and micro-gliosis (Zoli et al. 1999). Hence, future experiments assessing neuroinflammatory cytokines in α7 nAChR-knockout mice, or pharmacological use of α7 nAChR-specific agonists in VChT-deficient mice would further our understanding on how central cholinergic activity modulates inflammatory responses.

We have demonstrated that we are able to successfully express hM3Dq DREADDs specifically in microglia in mice. These early but important findings suggest that we could utilize DREADD technology to specifically activate microglia in the brain without the peripheral inflammatory responses effects observed when using LPS injections. Future experiments should address whether activating microglia using CNO elicits response through up regulation of anti-inflammatory cytokines. The innate immune response has been suggested to play a critical role in AD pathology (Heppner et al. 2015). Given that we could successfully express hM3Dq in microglia, it would be critical to address the inflammatory response of Gq activation of these cells using CNO. Given that M3 mAChRs are upregulated in adult cortical microglia treated with interferon-gamma (Pannell et al. 2016), it seems of importance to investigate how activation of Gq signalling in microglia influences inflammatory responses. GPCR signalling has been implicated in regulating the expression of inflammatory genes, including nuclear factor-κB (NF-κB), which regulates a number of inflammatory cytokines (Ye 2001). Indeed, it has been demonstrated that NF-κB, which is
expressed in microglia (Qin et al. 2005), plays a critical role in hippocampal synaptic plasticity (Albensi and Mattson 2000; Meffert et al. 2003). Hence, it is tempting to hypothesize that activation of Gq signalling in microglia through CNO induces an anti-inflammatory response or inhibits pro-inflammatory cytokine release. Findings of these experiments will lay the groundwork for investigation of the effects of central microglial activation on the innate immune system and the consequences that may have on neuropathology and cognition in mouse models of AD.

4.3 Cholinergic deficiency and adult hippocampal neurogenesis

Adult hippocampal neurogenesis has been associated with a number of learning and memory processes (Leuner et al. 2006; Aimone et al. 2009). An increase in number and survival of newly born cells in the hippocampus has been reported after exercise (van Praag et al. 1999), and hippocampal-dependent learning tasks (Gould et al. 1999). Administration of cholinesterase inhibitors, such as galantamine, for 14 days significantly increase the number of BrdU-positive cells in the dentate gyrus in the adult mouse brain (Jin et al. 2006). Moreover, selective lesions in rats using 192 IgG-saporin significantly increased apoptosis in the dentate gyrus (Cooper-Kuhn et al. 2004). Separate basal forebrain 192 IgG-saporin lesion experiments in rats, however, suggest that cholinergic
signalling is critical for cell proliferation rather than long-term survival (Mohapel et al. 2005). Thus, the exact role of cholinergic signalling in regulating cell proliferation and survival in the hippocampus is unclear. Our data support a role for cholinergic signalling in proliferation and differentiation of newborn cell survival in the hippocampus.

Previous work has demonstrated the involvement of cholinergic signalling in regulating adult hippocampal neurogenesis via mAChRs. For example, increase in cell proliferation in the SGZ post-administration of galantamine is blocked by scopolamine (muscarinic receptor antagonist) (Kita et al. 2014). Interestingly, this effect was also observed after administration of telenzepine, an M1 mAChR subtype antagonist, but not by mecamylamine (nAChR antagonist) nor the α7 nAChR specific antagonist methyllycaconitine (Kita et al. 2014). Similarly, the authors showed an enhancement in cell proliferation in the dentate gyrus after administration of oxotremorine, a muscarinic agonist (Kita et al. 2014). Indeed, data from separate studies demonstrate that M1 and M4 mAChRs are expressed on newly formed cells in the dentate gyrus 24 hours-post BrdU injections (Mohapel et al. 2005). Interestingly, impairments in cell proliferation induced by forebrain 192 IgG-saporin lesions in rats were reversed by chronic activation of M1 mAChRs (Van Kampen and Eckman 2010). These findings were accompanied by the finding of an increase in the number of neurons expressing a neuronal marker, suggestive of enhanced number of newly generated cells (Van Kampen and Eckman 2010). Together, these findings support the notion
that the increase in cell proliferation reported after administration of cholinesterase inhibitors is mediated, at least in part, by activating M1 mAChRs. Hence, M1 MAChRs may serve as a potential target to reverse the impairments in adult neurogenesis in VAcT mutant mice.

These pro-proliferative effects of mAChRs may involve the activation of downstream signalling pathways, including mitogen-activated protein kinases (MAPK) and PI-3 kinases. Indeed, muscarinic receptors expressed by neural precursor cells enhance DNA synthesis in neural precursor cells via the Ras pathway, which leads to phosphorylation of MAPK (Crespo et al. 1994; Ma et al. 2000). Similarly, in vitro experiments demonstrate that carbachol (non-selective cholinergic agonist)-mediated increase in [3H]thymidine levels was substantially attenuated when a PI-3K inhibitor was used, suggesting a critical role for PI-3K signalling in neural precursor cell proliferation (Li et al. 2001). Relevant targets of PI-3K include GSK3, which phosphorylates β-catenin, a critical component of the canonical Wnt/β-catenin pathway (Nelson and Nusse 2004). Activation of this pathway promotes neural precursor differentiation, while inactivation impairs differentiation (Lie et al. 2005). Further evidence shows that cholinergic signalling via α7 nAChRs may also plays a critical role in cell survival, but not cell proliferation (Kita et al. 2014). Activation of the α7 nAChR using PHA-543613 enhanced cell survival in the dentate gyrus of adult mice, an effect that was not seen with donepezil injections (Kita et al. 2014). These observations indicate that cholinergic neurotransmission, through distinct muscarinic and nicotinic
receptors, could modulate cell proliferation and survival in the adult hippocampus. Our data further support a role for ACh in hippocampal neurogenesis, whether the adult hippocampal neurogenesis deficits reported in VACHT-deficient mice are mediated by M1 MACHRs and/or α7 nACHRs remains to be established.

Cholinergic signalling could regulate neurogenesis by affecting the levels of growth factors, including brain-derived neurotrophic factor (BDNF), which is critical for adult hippocampal neurogenesis in mice (Lee et al. 2002). For example, administration of galantamine in mice enhances phosphorylation of BDNF and trkA (a nerve growth factor receptor) in the hippocampus (Autio et al. 2011). Moreover, rats with 192 IgG-saporin lesions displayed a decrease in proBDNF protein expression, which is the precursor of BDNF (Gil-Bea et al. 2011). Interestingly, galantamine injections were sufficient to rescue the decrease in proBDNF protein expression, suggesting that cholinergic tone may regulate neurotrophic factor signalling in the adult brain (Gil-Bea et al. 2011). In addition to BDNF, the insulin-like growth factor 2 (IGF2) is highly expressed in neural precursor cells, and through AKT signalling, regulates neural cell proliferation in the hippocampus of mice (Bracko et al. 2012). Indeed, experiments demonstrate that galantamine selectively enhances IGF2 protein levels in the hippocampus, but not the cortex, in adult mice (Kita et al. 2013). Interestingly administration of methyllycaconitine, which is an α7 nACHR antagonist, blocks the increase in IGF2 protein expression suggesting that
enhancement of cholinergic tone using galantamine enhances IGF2 expression via \( \alpha_7 \) nAChR signalling in the hippocampus (Kita et al. 2013). Our findings indicate that deficient cholinergic signalling has profound consequences on hippocampal neurogenesis. Because of the involvement of cholinergic tone in regulating neurotrophic factors, further experiments are warranted to address if the deficits in hippocampal neurogenesis in forebrain VAcT-deficient mice occur via the down regulation of neurotrophic factors in the hippocampus.

Our findings demonstrate that impairments in neurogenesis have a functional consequence for pattern separation as assessed in the two-choice discrimination touchscreen task. A number of studies have demonstrated the role of the dentate gyrus in pattern separation (Gilbert et al. 2001; Sahay et al. 2011). The synaptic transmission pathway thought to mediate pattern completion is via the entorhinal cortex to the dentate gyrus, and ultimately to the CA3 (Treves and Rolls 1994; Leutgeb et al. 2007; Bakker et al. 2008). Indeed, lesions to the dentate gyrus impair pattern separation in rats (Gilbert et al. 2001; Kesner et al. 2004). Furthermore, electrophysiological recordings in rats have provided insight regarding the specific role of the dentate gyrus in pattern separation. Leutgeb et al. recorded from active hippocampal cell assemblies in rats that were in either a square box with flexible walls that gradually transformed into a circular box (i.e. small pattern separation), or in two or three different enclosures in a separate room setting (i.e. large separation) (Leutgeb et al. 2007). Within an active cell assembly, the dentate gyrus and the CA3 regions of the hippocampus were
highly active when the environment was slightly modified (small separation) (Leutgeb et al. 2007). Interestingly however, when a large separation was presented (i.e. change of rooms), only the CA3 but not the dentate gyrus, displayed higher activity within an active cell assembly (Leutgeb et al. 2007), suggesting a critical role of the dentate gyrus in pattern separation. Conversely, ablation of adult hippocampal neurogenesis in the SGZ in mice using low-dose x-irradiation impaired pattern separation in a touchscreen based-task (Clelland et al. 2009). Interestingly, mice with ablated neurogenesis were only impaired when stimuli were presented with a small separation, but not when a high separation was presented, suggesting that NSCs in the dentate gyrus may be required for pattern separation (Clelland et al. 2009). Further experiments demonstrate that BDNF expression in the dentate gyrus is required for pattern separation (Bekinschtein et al. 2014). It is important to note that VACHT-deficient mice exhibit an array of cognitive abnormalities even at a young age, and that these abnormalities may interfere with performance in pattern separation. However, because VACHT-deficient mice were able to perform comparably to controls on the large separation, it is possible that the neurogenesis deficit may underlie their poor performance in the small separation.

The involvement of cholinergic signalling in pattern separation is not fully understood, however, it appears that cholinergic neurotransmission is required for maintenance of the dentate gyrus-CA3 connections (Barnes et al. 2000). Additionally, immature hippocampal granular cells express M1 mAChRs and play
a critical role in enhancing neural precursor cell proliferation (Itou et al. 2011). Moreover, septal cholinergic tone has been implicated in modulating the excitability of neural precursor cells by enhancing their firing properties (Vogt and Regehr 2001). Indeed, experiments show that the basal firing rate of cells in the dentate gyrus is low, and is highly enhanced by muscarine (Vogt and Regehr 2001). This cholinergic-mediated enhancement of neuronal firing rates would ultimately facilitate information output to the CA3. Moreover, there has been evidence suggesting that NMDA plasticity is required for pattern separation. Mice with genetic deletion of the NR1 subunit of the NMDAR specifically in the dentate gyrus exhibited impairments in discriminating two similar contexts in contextual fear discrimination learning (McHugh et al. 2007). Thus, it would be critical to elucidate the type of synaptic plasticity that may underlie these cholinergic-mediated impairments in pattern separation observed in VACHT-deficient mice.

4.4 Central cholinergic control of locomotor activity

Our findings demonstrate that forebrain-specific VACHT knockout mice are significantly hyperactive compared to controls in a novel environment, and in a 24-hour metabolic cage. These findings supported our previous observations from our laboratory in a separate mouse line of VACHT forebrain cholinergic deficiency (Martyn et al. 2012). Importantly, our data demonstrating that elimination of ChAT in the striatum has no effect on locomotor activity are in line with our previous findings (Guzman et al. 2011), suggesting cortical/hippocampal
Cholinergic regulation of locomotion. Furthermore, the hyperactivity observed in VACht mutant mice support previous findings that show exploration in a novel environment results in ACh release in the cortex and hippocampus (Acquas et al. 1996). Interestingly, however, mice with threefold increase in ACh release in the hippocampus present normal locomotor activity (Kolisnyk et al. 2013). In an attempt to dissect the region involved in mediating the cholinergic-induced hyperactivity, we injected an adeno-associated virus AAV8-Cre or control virus AAV8-GFP into the medial septum/vertical limb of the diagonal band of VACht^flox/flox animals to selectively decrease VACht levels in the hippocampus (not shown; Gustavo Parfitt). Data from this study indicated that selectively decreasing VACht in the hippocampus showed a modest effect towards increasing locomotor activity, but no statistical significance was reached, compared to AAV8-GFP injected controls (not shown; Gustavo Parfitt). Hence, our data in the VACht^Nkx2.1-Cre-flox/flox mouse model support the notion that perhaps both NBM and septohippocampal cholinergic signalling are required to induce hyperactive behaviours.

Cholinergic dysfunction has been associated with many of the cognitive symptoms observed in schizophrenia (Sarter and Bruno 1999; Heimer 2000). Moreover, cholinergic deficiency has been implicated in modulating the activity of dopaminergic neurons (Cachope et al. 2012; Threlfell et al. 2012), which in turn elicits hyperactive behaviours (Mattsson et al. 2002; Patel et al. 2012). Studies in rats with selective ablation of basal forebrain cholinergic neurons using 192-IgG-
saporin demonstrate that they also present hyperactivity (Mattsson et al. 2002). In contrast, others have reported that using 192-IgG-saporin showed a very modest or no effect on locomotor activity (Leanza et al. 1995; Walsh et al. 1995). The inconsistency in the reports using immunotoxins to study the role of the basal forebrain cholinergic system could be due to the variability in size and location of the lesions; as well as differences in the degree of ablation. Hence, using immunolesion approaches to dissect the involvement of basal forebrain cholinergic tone may not be ideal. Hyperactive behaviour has also been associated with increased central glutamatergic activity. Indeed, mice with 192-IgG-saporin lesions to the basal forebrain show increased NMDAR activity in the CA1 region of the hippocampus (Jouvenceau et al. 1997). Moreover, stimulation of NMDARs in the hippocampus enhances locomotor activity via dopamine receptor 1 (D1) and D2 receptor activity in the nucleus accumbens (Wu and Brudzynski 1995; Bardgett and Henry 1999). Indeed, rats with 192-IgG-saporin lesions to the basal forebrain show enhanced sensitivity to amphetamine (Mattsson et al. 2002). Because amphetamine acts by inhibition of dopamine uptake (Heikkila et al. 1975), the increased sensitivity to amphetamine in mice with ablation of cholinergic neurons could be due to alterations in dopaminergic levels. In line with these studies, mice with genetic targeting of forebrain ChAT neurons show increased phasic-to-tonic dopamine signalling from the striatum, and display hyperactive behaviour (Patel et al. 2012). Whether the effect we observed in our mice involves enhanced cholinergic-mediated enhancement of dopaminergic activity remains to be established.
Evidence from several studies highlighted the involvement of central muscarinic receptors in regulating locomotion. For instance, mice lacking the M1 mAChR subtype exhibit hyperactive behaviour with no changes in anxiety-related behaviours, compared to controls (Miyakawa et al. 2001). Intriguingly, mice lacking the M2 and M3 mAChR subtypes present normal locomotor activity (Gomeza et al. 1999; Yamada et al. 2001), while mice lacking M4 mAChR subtype exhibit only mild hyperactivity compared to the M1 knockout mice (Gomeza et al. 1999; Miyakawa et al. 2001). Hence, future studies involving the hyperactive behaviour observed in our mice should involve investigating whether this phenotype is mediated by M1 mAChR activity. While the involvement of cholinergic signalling in locomotion has been examined, the contribution of cholinergic input from distinct brain regions remains unclear. For example, optogenetic silencing of cholinergic neurons in the caudate putamen or the nucleus accumbens has no effect on locomotor activity (Witten et al. 2010; English et al. 2012). Conversely, previous studies showed that depletion of striatal cholinergic cells using immunotoxin-mediated cell targeting caused hyperactive behaviour in mice (Kaneko et al. 2000). Given that striatal cholinergic interneurons co-release glutamate (Higley et al. 2011), the lack of effect on motor activity observed in their studies could be attributed to spared glutamatergic neurotransmission, as VGLUT3-knockout mice are hyperactive (Gras et al. 2008). Hence, it is of importance to investigate how cholinergic signalling
influences other neurotransmitters involved in controlling locomotor activity, including glutamate and dopamine.

4.5 Role of hippocampal cholinergic tone in paired associates learning

In this thesis we uncovered a role for forebrain cholinergic signalling in the acquisition of paired associates learning (PAL). We tested our mice using the operant touchscreen technology (Bussey et al. 2001; Talpos et al. 2009; Bartko et al. 2011), which is analogous to tests used in humans. Previous work using a pairwise discrimination task touchscreen system has demonstrated that inhibition of forebrain cholinergic signalling has no effect on acquisition of the task, but impairs reversal learning when contingencies are switched (Kolisnyk et al. 2013). Interestingly, overexpression of VAChT in mice impairs attention in a 5CSRTT touchscreen task (Kolisnyk et al. 2013). These previous findings suggest a critical role of cholinergic signalling in executive functions. However, the involvement of hippocampal cholinergic tone in the acquisition of PAL is still under debate. Systemic administration of donepezil after acquisition of PAL significantly improved PAL performance in mice, an effect that was attenuated with administration of muscarinic antagonists (Bartko et al. 2011). Similar results have been observed in monkeys as 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 signalling
might be relevant for PAL. Also, rats previously trained in PAL that received injections into the dorsal hippocampus of either scopolamine or mecamylamine and were re-tested did not show deficits in performance (Talpos et al. 2009), suggesting that hippocampal cholinergic signalling might not modulate recall in this task.

Our data demonstrate that selective inhibition of cholinergic signalling in the forebrain in mice leads to disruption of synaptic plasticity and specific cognitive impairments. In particular, we show that forebrain cholinergic neurotransmission is required for acquisition of the PAL task. The PAL task has been clinically used as a potential cognitive marker of decline in psychosis (Wood et al. 2002). In patients with schizophrenia, impairments were seen in the PAL task, which correlated with negative symptoms (Barnett et al. 2005). Moreover, the PAL task has also been considered a sensitive task for predicting cognitive decline in AD (Swainson et al. 2001; Blackwell et al. 2004). Our results indicate that disruption in forebrain cholinergic tone disturbs PAL learning. 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 deficient cholinergic tone. Interestingly, mice deficient for the M1 receptor were not impaired in the PAL task (Bartko et al. 2011).

PAL acquisition has been demonstrated to be associated with hippocampal function in humans and rats (Talpos et al. 2009; de Rover et al. 2011). For
example, inactivation of the rat hippocampus using lidocaine (non-selective Na\(^+\) channel blocker) significantly impairs performance post-acquisition of the PAL task, suggesting that the hippocampus is required 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 behavioural plasticity has been shown in rats with unilateral hippocampal lesions (Zou et al. 1999). To specifically address the contribution of hippocampal cholinergic tone to PAL performance, we injected VChT\(^{\text{floxflox}}\) mice with AAV8-Cre or AAV8-GFP virus injected to the medial septum and vertical limb of the diagonal band and tested their performance on the PAL task. We determined that hippocampal VChT levels positively correlated to PAL
performance (Al-Onaizi et al. 2016). These data 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. Taken together, our findings 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.

4.6 Cholinergic control of spatial navigation

We showed that acquisition of the spatial version of the MWM and recall of platform location was mildly affected in VACHT<sup>Nkx2.1-Cre-flox/flox</sup> mice. Interestingly, impairments in the spatial version of the MWM have been observed in rats with combined lesions of MS/VDB and 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). Similarly, data from AAV8-Cre injected mice did not show any deficit in this behavioural task (Al-Onaizi et al. 2016). Also, rats with 192 IgG-saporin lesions restricted to NBM did not show behavioural impairments in the MWM (Pizzo et al. 2002). These data suggest that forebrain cholinergic signalling 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 in order to produce a severe spatial deficit. Thus, providing that cortical cholinergic projections are intact, hippocampal cholinergic activity is not absolutely required for this behavioural 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) contribute to regulation of spatial memory by cholinergic neurons.

In contrast to reference memory in the MWM, VAChT\textsuperscript{Nlx2.1-Cre-flox/flox} mice presented extensive deficits in the MWM reversal-learning task, suggesting a prominent role for forebrain cholinergic signalling in reversal learning. Cognitive flexibility is an important domain for survival. It refers to the ability to adapt goal-directed behaviour in response to changing environmental conditions. Deficits in performance in tasks testing cognitive flexibility have been reported in early AD (Traykov et al. 2007; Petrova et al. 2010). Our findings are in line with another mouse line from our laboratory with selective deletion of forebrain-VAChT, which displayed impairments in the reversal protocol of the MWM, while showing no impairments in the acquisition (Martyn et al. 2012). Moreover, mice with forebrain-specific VAChT deletion displayed deficits in reversal learning in the touchscreen visual discrimination task (Kolisnyk et al. 2013).

In an attempt to dissect the contribution of hippocampal cholinergic tone to reversal learning performance, we assessed mice with selectively decreased VAChT expression in the medial septum (AAV8-Cre) in the MWM reversal-
learning task. These mice presented impairments in the MWM reversal-learning task, suggesting a prominent role for septohippocampal cholinergic signalling in reversal learning (Al-Onaizi et al. 2016). To account for compromised striatal cholinergic signalling in VACHT\textsuperscript{Nkx2.1-Cre-flox/flox} mice for the performance in the MWM, we also tested a mouse line with selective deletion of VACHT in striatal neurons (VACHT\textsuperscript{D2-Cre-flox/flox}), but spared hippocampal VACHT (Guzman et al. 2011). Interestingly, VACHT\textsuperscript{D2-Cre-flox/flox} mice did not differ from controls (VACHT\textsuperscript{flox/flox}) in both acquisition and reversal versions on the MWM (Al-Onaizi et al. 2016). Together with other findings from our laboratory, our data suggest that deficits seen in reversal learning in VACHT\textsuperscript{Nkx2.1-Cre-flox/flox} mice are not likely due to impaired striatal cholinergic transmission, but rather due to deficient hippocampal cholinergic tone. Nonetheless, it remains possible that NBM-cortical cholinergic inputs play an important role in modulating cognitive flexibility.

In terms of cognitive flexibility, the contribution of cholinergic signalling from the NBM still remains unclear. For example, NMDA-induced excitotoxic lesions of the NBM in marmosets, which decreased expression of acetylcholinesterase in the prefrontal cortex to over 70%, had a mild effect on acquisition but produced impairments in reversal learning on a visual discrimination task, suggesting a critical role of NBM in behavioural flexibility (Roberts et al. 1990). Conversely, ME20.4 IgG-saporin lesions to the NBM in marmosets showed no impairments in reversal learning in a simple visual discrimination task (Fine et al. 1997). Impairments, however, were observed after administration of scopolamine (Fine
et al. 1997), suggesting muscarinic receptor involvement in cognitive flexibility. In rats, infusion of non-selective quisqualic acid to lesion the NBM led to impairments in reversal learning in an olfactory discrimination task (Bailey and Thomas 2001). Similarly, infusions of 192 IgG-saporin into the NBM in rats selectively impaired serial reversal learning in an operant chamber, while performance on the first reversal was preserved (Cabrera et al. 2006). On the other hand, 192 IgG-saporin lesions to the NBM in rats had no effect on performance in reversal learning in an olfactory discrimination task (Bailey et al. 2003). Although IgG-saporin is selective in targeting cholinergic neurons, the differences in findings regarding cognitive flexibility would suggest that reversal learning could be dependent on a population of non-cholinergic cells located in the NBM (Tait and Brown 2008), or a combination of both (Dunnett et al. 1987; Bailey et al. 2003). From our findings (Al-Onaizi et al. 2016), it appears that input to the hippocampus regulates cognitive flexibility; however, we cannot exclude the possible involvement of NBM-cortical signalling in these tasks. Further experiments are needed to determine the role of NBM-cortical cholinergic signalling in cognitive flexibility.

The use of cholinergic antagonists has been shown to alter cognitive flexibility. For example, systemic administration of scopolamine, a muscarinic receptor antagonist, impaired set shifting and reversal learning in rats while having no effect on discrimination performance (Chen et al. 2004). Similar findings have been reported using systemic injections of scopolamine in rats, where a dose 0.5
mg/kg was sufficient to significantly impair rats’ reversal performance, while visual discrimination remained similar to controls (Soffie and Lamberty 1987). In addition, systemic injections of CDD-0102A, a selective M1 muscarinic agonist, had a significant effect on inhibition of a previously relevant strategy and acquire a new strategy in a strategy-shifting task in rats (Ragozzino et al. 2012). Administration of this drug however had no effect on acquisition of a place or visual cue discrimination (Ragozzino et al. 2012), suggesting that central muscarinic receptor activity is critical in mediating reversal learning. Nonetheless, M1 knockout mice showed normal reversal learning in the visual discrimination task (Bartko et al. 2011) suggesting that other muscarinic receptors and/or nicotinic receptors are involved in facilitating reversal learning. In line with our results, these findings suggest that cholinergic tone plays a critical role in cognitive flexibility, however the involvement of the cholinergic receptor subtypes needs to be elucidated.

We provided evidence that cholinergic dysfunction impairs hippocampal LTP. Synaptic plasticity is considered the molecular correlate of learning and memory (Morris et al. 1986; Morris 1989; Buzsaki 2005). Our findings are in line with previous observations from our laboratory, which demonstrated that ex vivo LTP is impaired in mice with 70% decrease in VACht levels, and in mice with forebrain deletion of VACht (Martyn et al. 2012). These studies however, were performed in hippocampal slices ex vivo, hence cholinergic terminals were not activated (Martyn et al. 2012). Our current findings provide further evidence that
Schaffer collateral-CA1 synapses-mediated LTP requires intact cholinergic input. Previous observations have highlighted a critical role of cholinergic tone to hippocampal synaptic plasticity. For example, *in vitro* studies using cholinergic agonists significantly enhance LTP (Blitzer et al. 1990). Moreover, studies in anaesthetized rats show that tetanic stimulation of the medial septum induces hippocampal LTP, an effect that was blocked by atropine (muscarinic antagonist) (Markevich et al. 1997). Also, experiments in wild-type walking rats reveal an increase in LTP induction in the hippocampus compared with immobility, an effect that was abolished in mice with 192 IgG-saporin immunotoxin lesions of the medial septum (Leung *et al.* 2003). In line with our findings, these data suggest that septohippocampal cholinergic tone plays a critical role in modulating hippocampal plasticity.

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). Experiments have demonstrated that the induction of LTP and STD in Schaffer collateral-CA1 synapses is dependent on α7 nAChR activation, and is timing-dependent (Gu and Yakel 2011). In particular, ex vivo studies show that in hippocampal slices of α7 nAChR-knockout mice, both presynaptic and postsynaptic α7 nAChR activation is required for LTP and STD (Gu et al. 2012). This suggests that cholinergic neurotransmission via both pre- and post-synaptic α7 nAChRs regulates hippocampal synaptic plasticity. α7 nAChR-mediated synaptic plasticity involves α7 nAChR activation of NMDARs. Indeed, induction of α7 nAChR-mediated LTP requires the activation and prolongation of calcium transients in NMDARs in CA1 pyramidal neurons (Gu and Yakel 2011). The impairments observed in VAcT^{Nkx2.1-Cre-flox/flox} mice in LTP suggest that cholinergic signalling may regulate NMDAR-mediated synaptic plasticity.

4.7 Future Studies

Our findings regarding neurogenesis impairments in VAcT-deficient mice suggest that cholinergic tone regulates proliferation and cell survival of neural precursor cells in the hippocampus. Because neurotrophic factors play a critical role in adult hippocampal neurogenesis (Bekinschtein et al. 2014) future studies should involve investigating the levels of distinct neurotrophic factors such as BDNF, which is involved in enhancing neurogenesis by increasing dendritic spine reorganization in the rat hippocampus (Pencea et al. 2001; Scharfman et al.
2005; Kramar et al. 2012). Furthermore, it would be of interest to dissect the involvement of muscarinic receptor activity in regulating neurogenesis. Because M1 and M4 mAChRs are expressed early in neuroblasts (Mohapel et al. 2005), potential experiments to rescue neurogenesis deficits should involve using M1 or M4 mAChR agonists in VACHT-deficient mice. Importantly, it would be critical to investigate if mAChR agonists or cholinesterase inhibitors would improve pattern separation in these cholinergic-deficient mice. These experiments would further our understanding regarding the specific cholinergic receptors involved in the regulation of adult hippocampal neurogenesis. Alternatively, it would be intriguing to investigate whether exercise, which enhances adult hippocampal neurogenesis, would have any significant impact on rescuing the neurogenesis deficits in VACHT-deficient mice.

We demonstrated that forebrain cholinergic signalling is required for acquisition in the paired associates learning touchscreen task in Chapter 3.4.1. These data suggest that forebrain cholinergic tone is required for PAL, a task which MCI patients are impaired in (Keri et al. 2012). Moreover, experiments from our laboratory demonstrate that VACHT levels correlate with performance in PAL (Al-Onaizi et al. 2016). Because the use of cholinesterase inhibitors has shown promising effects on reducing the annual rate of hippocampal atrophy in prodromal AD (Dubois et al. 2015), experiments involving administration of donepezil in mice with selective decrease of VACHT (AAV8-Cre mice) would be important. These experiments would address if donepezil has a positive effect in
rescuing the cognitive impairments observed in VACHT deficient mice. Because the mouse version of the PAL touchscreen task is analogous to the human version, results from these experiments regarding the effects of donepezil of PAL performance will be highly translatable to humans.

We demonstrate in this thesis that forebrain cholinergic signalling is required for cognitive flexibility. Moreover, viral injection experiments from our laboratory showed a critical role for septohippocampal cholinergic tone in behavioural flexibility (Al-Onaizi et al. 2016). However, the exact involvement of the NBM-cortical signalling to these behaviours remains unclear. Viral injections to decrease VACHT levels in the NBM would aid elucidate the contribution of NBM cholinergic signalling to behavioural flexibility. NBM AAV8-Cre injected mice would not only be useful for assessment of behavioural flexibility, but could also help in determining the role of NBM cholinergic signalling in PAL, and locomotor activity.

4.8 Significance of Research and Conclusions

Neurodegeneration of basal forebrain cholinergic neurons constitute a major hallmark in the AD. In addition, long-term use of drugs with anti-cholinergic side effects significantly increases an individual’s risk of developing AD (Gray et al. 2015; Risacher et al. 2016). Although the cholinergic system remains the main
target in treating AD symptoms, the efficacy of cholinesterase inhibitors has been questioned (Kaduszkiewicz et al. 2005; Takeda et al. 2006). Therefore, new potential targets need to be established as well as potential biomarkers to aid the diagnosis and track disease progression. The data presented in this thesis show how alterations in this system influences distinct cognitive behaviours, including the PAL task, and adult hippocampal neurogenesis. As PAL performance may be dependent on cholinergic integrity, the PAL task could be used to identify individuals with cognitive dysfunction linked to cholinergic abnormalities. In this thesis we also provided evidence that our mouse model of cholinergic dysfunction mimics an array of behavioural and molecular abnormalities common to AD. Importantly, our data sheds light on how long-lasting cholinergic deficiency may relate to AD-like pathology.

Imaging studies involving volumetric measurement of basal forebrain cholinergic nuclei in humans reveal a drastic decrease in the volume of basal forebrain cholinergic neurons in AD and MCI patients, in comparison to healthy elderly controls (Grothe et al. 2010; Grothe et al. 2012; Grothe 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 behavioural consequences of cholinergic malfunction. The work presented in this thesis is relevant to understand how cholinergic dysfunction or degenerative changes in cholinergic
neurons contribute to cognitive alterations and neuropathology in several neurodegenerative disorders, including AD.
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Appendix A- Regulation of cognitive processing by hippocampal cholinergic tone

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*Equal contribution

Contributions to publication: M.A.A-O performed all immunohistochemistry and biochemical analyses in Nkx2.1-Cre;tdTomato and VACHTNkx2.1-Cre-flox/flox mice. M.A.A-O performed cognitive (PAL, MWM) and behavioural analyses (neuromuscular testing) in VACHTNkx2.1-Cre-flox/flox mice.
 ORIGINAL ARTICLE

Regulation of Cognitive Processing by Hippocampal Cholinergic Tone

Mohammed A. Al-Onaizi¹,²,†, Gustavo M. Parfitt¹,⁵,†, Benjamin Kolisnyk¹,³, Clayton S. H. Law⁴, Monica S. Guzman¹,⁴, Daniela Martí Barros⁵, L. Stan Leung⁴, Marco A. M. Prado¹,²,³,⁴ and Vania F. Prado¹,²,³,⁴

¹These 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 Yake 2011). Septal cholinergic activation, either by electrical stimulation or by optogenetics, allows the expression of distinct time-dependent actions in hippocampal plasticity (Gu and Yake 2011). Pharmacological (Decker and Majchrzak 1992; Anagnostaras et al. 1999; Gallet et al. 2001; Wallenstein and Vago 2001; Chudasama et al. 2004; Pitch 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 septo-hippocampal 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; Kolinsky, Guzman, et al. 2013).

The recent development of automated touch-screen 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; Remberg 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<sup>lox/lox</sup> mice was previously described (Martins-Silva et al. 2011). VACHT<sup>fl/ox</sup> mice (mixed C57BL/6J × 129/SvEv background, backcrossed to C57BL/6J for 5 generations) were crossed to VACHT<sup>fl/ox</sup> mice so that offspring from this mating provided control and test littermates. VACHT<sup>fl/ox</sup> mice were generated by crossing VACHT<sup>fl/ox</sup> 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<sup>fl/ox</sup> littermates. The reporter mouse line Nkx2.1-Cre/+ mouse line (C57BL/6J-Tg(Nkx2-1-cre)2sand/J), 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).

Immunofluorescence 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 1x 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 1x PBS (1 per well in a 24-well plate) were permeabilized with 0.4% Triton X-100 in 1x PBS for 1 h. Non-specific epitopes were blocked using a solution of 1x 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, Bibo, 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 x 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, the mouse was moved to the next stage, and a trial was done. 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 in one of the 3 screens (Must initiate; Phase 4). This was done until mice completed 36 trials in 60 min for 1 day. Next, animals go to the next 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+) 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 towards 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 (0.5 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, L 1.24 (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.

Sterotaxic 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 (AAV8-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^−/− 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 Bonferroni 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_{df} = 6.162, P = 0.0025), hippocampus (t_{df} = 4.461, P = 0.0097), and striatum (t_{df} = 8.625, P = 0.0010) were severely diminished in VACHT^Nkx2.1-Cre-fox/fox+ mice (see Supplementary Fig. 18-D). In contrast, VACHT levels remained unchanged in the brainstem of VACHT^Nkx2.1-Cre-fox/fox− compared with controls (t_{df} = 1.040, P = 0.3571, see Supplementary Fig. 1E). Moreover, immunofluorescence imaging indicated decreased VACHT immunoreactivity in the hippocampus of VACHT^Nkx2.1-Cre-fox/fox− 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-Cre-fox/fox− mice on the PAL task, which requires sophisticated processing of information for proper association of images with specific locations. VACHT^Nkx2.1-Cre-fox/fox− 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_{df} = 0.749) (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-Cre-fox/fox} 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^{fox/fox} (n = 3) and (B) VAChT^{Nkx2.1-Cre-fox/fox} 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^{fox/fox} (n = 7 clear squares) and VAChT^{Nkx2.1-Cre-fox/fox} (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 < 0.001).

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. 1E). VAChT^{fox/fox} mice (controls) were able to improve performance reaching 77 ± 1% accuracy by Week 9 (Fig. 1E). In contrast, peak accuracy performance of VAChT^{Nkx2.1-Cre-fox/fox} mice in the dPAL task during the same period was 58 ± 2% (Fig. 1D). Although VAChT^{Nkx2.1-Cre-fox/fox} 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. 1F). VAChT^{Nkx2.1-Cre-fox/fox} 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. 1F). 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. 1G). Furthermore, VAChT^{Nkx2.1-Cre-fox/fox} 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. 1H). In summary, VAChT^{Nkx2.1-Cre-fox/fox} 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-Cre-fox/fox} mice have intact synaptic plasticity, we examined LTP of the Schaffer collaterals on hippocampal CA1 neurons in anaesthetized mice in vivo. VAChT^{Nkx2.1-Cre-fox/fox} mice showed decreased LTP which lasted about 90 min post-tetanus delivery while LTP in VAChT^{fox/fox} mice was maintained for 120 min (Fig. 2A,B). This indicated that the lack of cholinergic signaling disturbs synaptic plasticity in hippocampal CA1 area in vivo.
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. 3E) or mean reward collection latency across the task (Pearson’s $r = 0.1799$, CI = -0.5676 to 0.7371, $P = 0.4352$, Fig. 3F), 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 VARCHT<sup>Nkx2.1-Cre-flx/flx</sup> mice compared with controls (see Supplementary Fig. 5A-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, $F_{3,39} = 39.58$, $P < 0.0001$, and an interaction effect $F_{3,39} = 2.914$, $P = 0.0394$, see Supplementary Fig. 5D), post hoc analysis revealed that both groups spent significantly more time in the target quadrant. However, VARCHT<sup>Nkx2.1-Cre-flx/flx</sup> mice had significantly fewer platform crosses compared with littersmate controls ($t_{29} = 2.795$, $P = 0.0112$, see Supplementary Fig. 5E).

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 VARCHT<sup>flx/flx</sup> mice (see Supplementary Fig. 6A,B). VARCHT<sup>flx/flx</sup> 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. 6E). 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, $t_{10} = 0.453$, $P = 0.665$, see Supplementary Figure 6C,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. 4A). 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. 4B). 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, $F_{3,39} = 37.81$, $P < 0.0001$, no effect of Cre expression, $F_{1,10} = 0.6452$, $P = 0.4237$, and no interaction $F_{3,104} = 0.3988$, $P = 0.7541$, Fig. 4D) or platform crosses ($t_{29} = 0.9547$, $P = 0.3603$, Figure 4E). Taken together, these results suggest that decreased levels of hippocampal cholinergic activity do not seem to affect MWM performance.

VAChT<sup>Nkx2.1-Cre-flx/flx</sup> 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<sup>Nkx2.1-Cre-flx/flx</sup> mice (2-way RM ANOVA shows a significant effect of days $F_{3,39} = 8.632$, $P = 0.0003$, main effect of genotype $F_{1,10} = 11.17$, $P = 0.0075$, and
no interaction \( F_{3,50} = 1.501, P = 0.2342, \text{Fig. 5A–C} \). 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 \( F_{3,80} = 3.133, P = 0.0301, \text{Fig. 5D} \), while VACHT\textsuperscript{Nikx2.1-Cre}\textsuperscript{lox/lox} mice visited all quadrants almost equally.
The number of platform crosses was also higher for control mice compared with VAChT mutants (t(20) = 2.797, P = 0.0111, Fig. 5E). These results indicate that, different from control mice, VAChT<sup>Nkx2.1-Cre-fl<sup>ox/</sup>fl<sup>ox</sup> 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<sup>Nkx2.1-Cre-fl<sup>ox/</sup>fl<sup>ox</sup></sup> mice for the performance in the MWM (see Supplementary Fig. 1D), we also tested a mouse line with selective deletion of VAChT in striatal neurons (VAChT<sup>D2-Cre-fl<sup>ox/</sup>fl<sup>ox</sup></sup>), but spared hippocampal VAChT (Guzman et al. 2011; see Supplementary Fig. 7). Interestingly, VAChT<sup>D2-Cre-fl<sup>ox/</sup>fl<sup>ox</sup></sup> mice did not differ from controls (VAChT<sup>fl<sup>ox/</sup>fl<sup>ox</sup></sup>) in both acquisition and reversal versions on the MWM (see Supplementary Fig. 7D–H). These results suggest that deficits seen in reversal learning in VAChT<sup>Nkx2.1-Cre-fl<sup>ox/</sup>fl<sup>ox</sup></sup> 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, \(F_{3,104} = 21.96, P < 0.0001\) and a significant interaction effect, \(F_{3,104} = 7.507, P = 0.0004\)), 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 VAChT-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 (\(t_{20} = 0.9547, P = 0.0010\), Fig. 5K). These results reveal that disruption of hippocampal cholinergic tone, but not striatal 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<sup>Nkx2.1-Cre::fox/fox</sup> 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<sup>Nkx2.1-Cre::fox/fox</sup> 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<sup>Nkx2.1-Cre::fox/fox</sup> mice have impaired working memory. Similarly, VAChT<sup>Nkx2.1-Cre::fox/fox</sup> 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 (\(t_{20} = 2.847, P = 0.0111\), Fig. 6D) and distance savings ratio (\(t_{20} = 2.149, P = 0.0473\), Fig. 6E). On the spontaneous alternations Y-maze task, AAV8-GFP-Cre-injected mice showed impairments on working memory, measured as a significant decrease of spontaneous alternations (\(t_{20} = 3.347, P = 0.0041\), 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<sup>Nkx2.1-Cre::fox/fox</sup> 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
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 activity was changed in hippocampal lesions in reversal learning (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 VACHTeNkx2.1-Cre-flx/fox1mice 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; Kolinsky, 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 VACHTeNkx2.1-Cre-flx/fox1mice, 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 VACHTeNkx2.1-Cre-flx/fox1 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 VACHTeNkx2.1-Cre-flx/fox1 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; Kolinsky, 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 NMDA 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 VACHTlox2.1-Cre-flx/lox 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 (VACHTlox2.1-Cre-flx/lox 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.

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Appendix B- Cholinergic surveillance over hippocampal RNA metabolism and Alzheimer’s-like pathology

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*Equal contribution

Contributions to publication: M.A.A-O performed tau Thr231 immunohistochemistry, Thioflavin-S staining, Congo red staining, activated caspase-3 immunohistochemistry, silver staining analysis, CD-68 immunohistochemistry, qPCR analysis of inflammatory markers, and Morris Water Maze analysis in young, and aged mice.
Cholinergic Surveillance over Hippocampal RNA Metabolism and Alzheimer’s-Like Pathology

Benjamin Kolisnyk1,2, Mohammed Al-Onaizi1,4, Lilach Soreq5, Shahar Barbash6, Uriya Bekenstein6, Nejc Haberman5, Geula Hanin6, Maxine T. Kish1,3, Jussemara Souza da Silva1, Margaret Fahnestock7, Jernej Ule5, Hermona Soreq6, Vania F. Prado1,2,3,4 and Marco A. M. Prado1,2,3,4

1 Robarts Research Institute, 2 Graduate Program in Neuroscience, 3 Department of Physiology and Pharmacology and, 4 Department of Anatomy and Cell Biology, Schulich School of Medicine and Dentistry, University of Western Ontario, London, Ontario, Canada N6A5K8, 5 Department of Molecular Neuroscience, UCL Institute of Neurology, Queen Square, London WC1N 3BG, UK, 6 The Edmond and Lily Safra Center for Brain Science and The Silberman Institute of Life Sciences, The Edmond J Safra Campus, The Hebrew University of Jerusalem, Jerusalem 91904, Israel and 7 Department of Psychiatry andBehavioural Neurosciences, McMaster University, Hamilton, Ontario, Canada L8S 4K1

Original Article

Abstract

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 Aβ42. This is accompanied by age-related increases in GSK3 activation, tau hyperphosphorylation, 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.

Key words: acetylcholine, Alzheimer’s disease, cognition, pathology, RNA metabolism
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 anticholinergic 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 hyperphosphorylation, 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.

Materials and Methods

Mouse Lines

Generation of VAChT<sub>lox/lox</sub> mice was previously described (de Castro et al. 2009). VAChT<sup>−/−</sup>Nkx2.1-Cre-lox/lox<sup>−/−</sup> mice were generated by crossing VAChT<sub>lox/lox</sub> (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 VAChT<sub>lox/lox</sub> 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.

RNA Sequencing

Mouse hippocampal tissue was rapidly dissected, and total RNA was extracted from individual samples using the PureLink RNA Mini Kit (Ambion). Two micrograms 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. Five 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 Array-Express (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-3897.

For human brains, we used the SQUARETM RNA library construction approach that utilizes different sets of 5′- and 3′-site specific primers to segregate all full-length transcripts into subpools 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 × 10<sup>8</sup> (STD = 2.0 × 10<sup>8</sup>) uniquely aligned 75 base pair (bp) single end reads or approximately 7.0 × 10<sup>6</sup> (STD = 1.8 × 10<sup>6</sup>) total read counts when combining all 12 SQUARE fields for each sample. These reads were mapped against the GRCh37/hg19 version of the Homo sapiens genome (http://genome.ucsc.edu/). Transcripts with >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 3). Expression criteria were set to RPKM > 1 in at least one of the SQUARE fields, in at least 80% of the tested donor cohorts.

Immunofluorescence

Immunofluorescence experiments were performed as previously described (de Castro et al. 2009). Primary antibodies used were anti-Cleaved caspase-3 (1:500 Thermo Fisher Scientific, Catalog no. P5-16335), anti-AT180 (1:1000 Thermo Fisher Scientific, Catalog no. EN-MN1040), and anti-PDSD95. Sections were visualized by Zeiss LSM 510 Meta (Carl Zeiss, Oberkochen, Germany) confocal system (<40, <63 objectives, with an N.A. of 1.3 and 1.4, respectively) and by Leica TCS S8P (Leica Microsystems Inc., Ontario, Canada) confocal system (<63 objective, with an N.A. of 1.4), a 488-nm Ar laser and 633-nm HeNe laser were used for excitation of fluorophores.

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).
Gene Ontology Analysis

Gene ontology functional analysis was performed using the GO-illia software through the web application. Using the 2 unranked lists method as described (Eden et al. 2009), KEGG pathway analysis was performed using the ClueGO plug-in of Cytoscape (Binde et al. 2009).

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 was then run through the Allen Brain Atlas (http://mouse.brain-map.org) to ensure that they were expressed in the murine hippocampus. All RNA-binding proteins not expressed in the hippocampus were excluded.

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.

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 10 μM of carbachol, 10 μM of carbachol and 100 μM Atropine, or 100 μM Atropine alone for 48 h.

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 50 mM Tris–HCl (pH 8.5); samples were then ultracentrifuged at 135 000 × g for 1 h at 4 °C; and the supernatant was collected and analyzed by western blotting and ELISA. The pellet was re-suspended and ultracentrifuged again and diluted in 8 M guanidine HCl to obtain the insoluble fraction for ELISA analysis.

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.

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.

Silver Staining

Assessment of argyrophilic cells in the hippocampus was done by using NeuroSilverTM staining kit II (FD NeuroTechnologies, Inc., Baltimore, MD, USA), which provides detection of degenerating neurons, including neuronal somata, axons, and terminals.

Estimation of Hippocampal Volume

NeuN immunohistochemistry was performed 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 dianobenzidine (Sigma, USA) and 0.5 mM H2O2 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.

GSK3 Inhibition

To inhibit GS3K in VACHTfl/fl 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, CA, USA). The pumps were implanted subcutaneously in the intrascapular 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/day of AR-A014418, a dose shown to produce a significant inhibition of GS3K 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.

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; Kolisnyk et al. 2013a). Briefly, animals were given 4 training trials a day (90 s each) for 4 days, with a 15-min intertrial 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 (>50% 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); and 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 1 strategy and 1 mouse.

Protein Isolation from Human Postmortem 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 (O斯塔penko et al. 2013). The samples were homogenized in RIPA buffer supplemented with protease inhibitor cocktail (Calbiochem), and Western blotting was performed as described above.

Statistical Analysis

Sigmastat 3.5 software was used for statistical analysis. Student’s t-test was used for comparison between 2 experimental groups. Two-way ANOVA or 2-way ANOVA with repeated measures (RM) were used when >2 groups were compared.

Results

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 VACHTfl/fl 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 (Prado et al. 2006; de Castro et al. 2009; Lima Rde et al. 2010). Non-biased, whole-genome transcriptome RNA sequencing of hippocampal samples from 3 VACHT-deficient and 4 control mice yielded a total of 14,200 expressed genes. Comparative analysis revealed that 1098 genes were differentially expressed in VACHTfl/fl mice compared with control mice (Fig. 1A,B, FDR corrected P < 0.05). Of those, 763 genes were upregulated and 362 downregulated in the VACHT-deficient mice. In addition, a linear regression analysis on reciprocal junction pairs detected roughly 4% of hippocampal transcripts in VACHTfl/fl mice as alternatively spliced in high confidence compared with control mice. Equal proportion of exon inclusion and exclusion events was observed; mainly events of cassette exons were detected (Fig. 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 PISK-Akt signaling pathway (a regulator of neuronal vulnerability (Gary and Mattson 2001; Endo et al. 2006)), splicingosome regulation, and regulation of microtubule-based processes were identified using Gene Ontology (GO) KEGG pathway analysis (Fig. 1D; see Supplementary Table 1). qPCR validation and correlation between changes observed in RNA-Seq and in an independent mouse cohort are shown in Supplementary Figure 1 for the different gene pathways and alternative splicing events. These results suggest that abnormal cholinergic signaling 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 (see Supplementary Fig. 2). VACHTfl/fl mice 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 (see Supplementary Fig. 2). 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.

Cholinergic Deficit Triggers Abnormal BACE1 Alternative Splicing and APP Processing

One of the detected abnormally alternatively spliced genes in our database was the protease BACE1 (Fig. 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 (Fig. 2A).

Bioinformatics analysis using the RBP-Map tool revealed an enrichment of binding sites for hnRNPA2/B1 in BACE1 mRNA. hnRNPA2/B1 is a 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 signaling are accompanied by decreased expression of hnRNPA2/B1 protein in the AD cerebral cortex and in cholinergic impaired mice (Bernson et al. 2012; Kolinsky et al. 2013b). Correspondingly, the hippocampus of VACHTfl/fl mice showed reduced hnRNPA2/B1 protein levels (Fig. 2B). We then investigated whether hnRNPA2/B1 regulates BACE1 splicing by exposing primary hippocampal cultured neurons to lentivirus-expressing lentiviral vectors (s) encoding BACE1-501. This decrease was blocked by cotreatment with the muscarinic antagonist atropine (Fig. 2C). These data implicate hnRNPA2/B1 in the regulation of BACE1 splicing.

To test for the role of cholinergic signaling 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 cotreatment with the muscarinic antagonist atropine (Fig. 2D). These data implicate muscarinic receptors in the regulation of BACE1 splicing. This splicing event in BACE1 predicts an increase in the levels of the AD marker APP and cleavage product p3 and p1 (Fig. 2D). The resulting APP fragment is predicted to increase APP levels in the hippocampus of VACHT-deficient mice (Fig. 2E).

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 VACHTfl/fl mice. Aged VACHT-deficient mice (11–14 month old) displayed a modified pattern of Tris-soluble APP fragments (Fig. 2F), similar to that of mouse models with APP/PS1 mutations (Oddo et al. 2003; Jankowsky et al. 2004). 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
were similar in VAChT-deicient mice and controls (Fig. 2G). APP processing was not modified in aged Nkx2.1-Cre mice (see Supplementary Fig. 3A), 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 VAChT-Nkx2.1-Cre-fox/fox mice showed increased levels of soluble mouse Aβ peptide compared with controls (Fig. 2H), 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-deicient mice, whereas it was highly abundant in the 5XFAD mice (Fig. 2H).
addition, neither control nor VACHT-deficient mice displayed positive Congo red staining, unlike brain sections from SxFAD mice, which exhibited numerous Congo red plaques (Fig. 21). 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 2 amino acid changes (Jankowsky et al. 2007).

**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 interrelated (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–fox/flox mice compared with aged-matched controls (Fig. 3A; 11–14 months old mice). To test whether 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 (aa 7–9) and C-terminal (313–333) amino acid sequences of tau, which is one of the earliest alterations of tau in AD (Wolozin et al. 1986; Weaver et al. 2000). Immunostaining with MC1 revealed positive immunoreactivity in the hippocampus of aged cholinergic-deficient mice, but not in age-matched controls (Fig. 3B).

In agreement with the immunofluorescence data, hippocampal extracts of VACHT\textsuperscript{Nkx2.1-Cre}\textsuperscript{+}\textsuperscript{lox}\textsuperscript{/}\textsuperscript{lox}\textsuperscript{-} mice showed approximately 4-fold increases in pTau immunoreactive bands, including higher order oligomers detected with AT180, compared with controls (Fig. 3C,D). On the other hand, total tau and pTauS262 levels were unmodified in VACHT-deficient mice (Fig. 3C,D). Taken together, our data indicate that deletion of hippocampal VACHT induces hyperphosphorylation 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 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\textsuperscript{Nkx2.1-Cre}\textsuperscript{+}\textsuperscript{lox}\textsuperscript{-} (right) mice. (n = 3, Scale bar, 100 μm). (B) Representative images of MC1 and Hoeschst labeling in the CA1 region of the hippocampus of controls (left) and VACHT\textsuperscript{Nkx2.1-Cre}\textsuperscript{+}\textsuperscript{lox}\textsuperscript{-} (right) mice. (n = 3, Scale bar, 100 μm). (C) Western blot analysis of controls (VAChT\textsuperscript{lox}\textsuperscript{-}\textsuperscript{-}) and VACHT\textsuperscript{Nkx2.1-Cre}\textsuperscript{+}\textsuperscript{lox}\textsuperscript{-} aged (11–14 months 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).
Aging causes a decline in spatial memory and learning ability. In the hippocampus, this failure leads to increased neuronal vulnerability and worsens cognitive functioning. Young VACHT-deficient mice (3–6 months old) used distinct strategies to reach the platform (Fig. 4E,F). However, aging significantly increased the number of activated caspase-positive cells in VACHT-deficient mice compared with controls (Fig. 4G). Also, young VACHT-deficient mice showed no alteration in the number of NeuN-positive cells across all regions of the hippocampus (Fig. 4G; see Supplementary Fig. 4); while NeuN-positive cells in CA1 and CA3 region, but not dentate gyrus, were decreased in aged VACHT-deficient mice (Fig. 4G; see Supplementary Fig. 4), predicting functional implications for this aging-related hippocampal neuronal vulnerability. Thus, impaired cholinergic signaling 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) showed little difference from controls in acquisition performance on the 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 4 days of acquisition (see Supplementary Fig. 4E,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 (Fig. 4H). At a young age, both controls and VACHT-deficient mice predominantly used a more direct strategy to reach the platform (strategies 5/6/7, Fig. 4I). In contrast, aged VACHT-deficient mice used random swimming predominantly as their strategy to acquire the task (strategy 2, Fig. 4I). While aged control mice maintained the use of more direct strategies, aged VACHT-deficient mice also exhibited deficits in the probe trial (see Supplementary Fig. 4G). Taken together, these results suggest that long-term cholinergic deficiency in VACHT-deficient mice led to progressive loss of neurons in the hippocampus that worsened spatial information acquisition and cognitive functioning.

Cholinergic-induced 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 signaling,
which has also been shown to play multiple roles in AD (Gary and Mattson 2001; Endo et al. 2006). As several genes that regulate the PI3-AKT pathway were upregulated in VAChT-deficient mice (Fig. 1D; see Supplementary Fig. 1), we tested for dysregulation of PI3-AKT signaling 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 VAChT<sup>Nkx2.1-Cre-ﬂox/ﬂox</sup> hippocampus compared with controls (Fig. 5A). Additionally, GSK3α/β tyrosine phosphorylation, which reﬂects activation of GSK3, was increased in these mutants (Fig. 5B). Hence, in addition to increased levels of proteins involved in AD pathology, these results suggest potential contributions of GSK3 activation in cholinergic-induced defects.

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) VAChT<sup>NKx2.1-Cre-ﬂox/ﬂox</sup> mice with the GSK3 inhibitor AR-A014418 (Fig. 5C). After 28 days of treatment, we found that VAChT-deﬁcient mice treated with AR-A014418 showed a signiﬁcant decrease in GSK3α and β tyrosine phosphorylation compared with VAChT<sup>Nkx2.1-Cre-ﬂox/ﬂox</sup> mice treated with saline (Fig. 5D). 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 VAChT<sup>Nkx2.1-Cre-ﬂox/ﬂox</sup> mice. AR-A014418 treatment was able to signiﬁcantly decrease

Figure 5. 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<sup>Nkx2.1-Cre-ﬂox/ﬂox</sup> 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 months old) controls (left) and VAChT<sup>Nkx2.1-Cre-ﬂox/ﬂox</sup> (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<sup>Nkx2.1-Cre-ﬂox/ﬂox</sup> mice. (D) Representative western blot and quantiﬁcation analysis of phospho-GSK3α and β in the hippocampus of aged VAChT<sup>Nkx2.1-Cre-ﬂox/ﬂox</sup> mice treated with AR-A014418 or saline. (E) Western blot analysis aged VAChT<sup>Nkx2.1-Cre-ﬂox/ﬂox</sup> treated with AR-A014418 or saline for Tau hyperphosphorylation at T231, MCI immunopositive tau, and total Tau protein expression. (F) Representative immunolabeling of reduced T231 Tau in the hippocampus of aged VAChT<sup>Nkx2.1-Cre-ﬂox/ﬂox</sup> mice (scale bar, 100 μm). (G) Western blot analysis for PSD-95 protein levels in the hippocampus of VAChT<sup>Nkx2.1-Cre-ﬂox/ﬂox</sup> mice treated with AR-A014418 or saline. (n = 4 saline treated, n = 5 AR-A014418 treated, data are mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001).
tau T231 hyperphosphorylation and MC1-immunopositive tau in Western blots (Chai et al. 2011; Petry et al. 2014), by approximately 50% in VACHT^NKx2.1-Cre-fox/fox mice compared with saline-treated VACHT^NKx2.1-Cre-fox/fox mice. Total levels of tau were unchanged (Fig. 5E). Immunofluorescence staining (Fig. 5F) also demonstrated reduced levels of T231 hyperphosphorylated tau in AR-A014418-treated mice. Compared with saline-treated animals, AR-A014418 treatment was able to significantly increase levels of PSD-95 protein (Fig. 5G).

Interestingly, we observed no changes in protein levels of BACE1 following AR-A014418 treatment in aged VACHTNKx2.1-Cre-fox/fox mice (see Supplementary Fig. 5A). Furthermore, AR-A014418 treatment did not alter the alternative splicing event in the BACE1 gene (see Supplementary Fig. 5A). 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, (see Supplementary Fig. 5C,D).

Cholinergic Dysfunction in Human AD Brains

Whether cholinergic genes are expressed in lower levels in human AD brain compared with 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 human AD brain compared with 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 Supplementary Table 2), including 8 sporadic AD patients and 16 from age-matched controls. We then profiled AD-related transcript differences by adopting the particularly deep SQUARE™ 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 with age-matched controls (Fig. 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 with age- and sex-matched controls (Fig. 6B), in agreement with previous observations (Efang et al. 1997; Chen et al. 2011). Furthermore, the cohort of AD brains exhibited 50% decrease in hnRNPA2/B1 protein levels compared with age-/gender-matched controls (Fig. 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 (Fig. 6D). AD brain samples also showed drastic increases in tau-Thr231 phosphorylation (Fig. 6E), which was inversely proportional to the levels of VACHT (Fig. 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 6. Cholinergic failure in human AD brain associates with loss of hnRNPA2/B1 and hyperphosphorylation of tau. (A) Cholinergic genes are downregulated 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 with 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).
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 suggest a causal role for cholinergic signaling 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 VACHT-Nkx2.1-Cre-fox/fox 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 (Tollervey et al. 2011; Berson et al. 2012; Bai et al. 2013). Importantly, spliceosome signaling 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 signaling 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; Kolinsky et al. 2013b) and hippocampus (Fig. 2B) leads to decreased expression of the hnRNPA2/B1 protein. Related work demonstrated that hnRNPA2/B1 is a cholinergic regulated splicing factor (Kolinsky et al. 2016). 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 by M1 muscarinic receptors (Kolinsky et al. 2016).

Cholinergic tone has been thought to regulate APP processing through muscarinic receptors (Nitsch et al. 1992; Davis et al. 2010). Specifically, M1 signaling 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 (Mower 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 that changes in alternative splicing occur both post-transcriptionally and independent of GSK3 signaling. 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, VACHT-Nkx2.1-Cre-fox/fox mice also show tau hyperphosphorylation, which destabilizes microtubules and significantly disrupts axonal transport. Tau hyperphosphorylation 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 hyperphosphorylation 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 overactivation 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 pathology was already present (11- to 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 result 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; Smale et al. 1995; Rogers and Shen 2000). 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 in Figure 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 have 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 hyperphosphorylation. Together with the mouse data, these observations support the notion that deficient cholinergic signaling in AD may correlate to key pathological events, including tau hyperphosphorylation.

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 (Naumann et al. 2002; Boskovic et al. 2014), 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 anticholinergic 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 whether 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 1-year treatment (Dubois et al. 2015). Our data point towards cholinergic signaling 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 than specific therapies based on reversing the individual processes it regulates.

Authors’ Contribution

B.K., M.A.A.O., L.S., J.U., H.S., V.F.P., and M.A.M.P. designed experiments; B.K., M.A.A.O., L.S., S.B., G.H., U.B., J.X., G.M.P., M.T.K., and J.S.S. conducted experiments; M.F. contributed with materials; B.K., M.A.A.O., L.S., G.H., U.B., and J.U. analyzed data; B.K., M.A.A.O., H.S., V.F.P., and M.A.M.P. wrote the manuscript. All authors read and approved the manuscript.
Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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Notes

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Conflict of Interest

The authors declare no competing financial interests.

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Appendix C- Cholinergic regulation of hnRNPA2/B1 translation by M1 muscarinic receptors.

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Contributions to publication: M.A.A-O performed hnRNPA2/B1 immunohistochemistry in VAChT-Nkx2.1-Cre-flox/flox mice, and in vitro hnRNPA2/B1 immunohistochemistry.
Cellular/Molecular

Cholinergic Regulation of hnRNPA2/B1 Translation by M1 Muscarinic Receptors

Benjamin Kolisnyk,1,2 Mohammed A. Al-Onaizi,1,4 Jason Xu,1,4 Gustavo M. Parfitt,1 Valeriy G. Ostapchenko,1,3 Geula Hanin,3,6 Hermona Soreq,3,6 Marco A. M. Prado,1,2,3,4 and Vania F. Prado1,2,3,4

Cholinergic vulnerability, characterized by loss of acetylcholine (ACh), is one of the hallmarks of Alzheimer’s disease (AD). Previous 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, heterogeneous nuclear ribonucleoprotein (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 used a combination of genetic mouse models and in vivo and in vitro techniques. Decreasing cholinergic tone reduced levels of hnRNPA2/B1, whereas increasing cholinergic signaling in vivo increased expression of hnRNPA2/B1. This effect was 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 signaling 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.

Key words: Alzheimer’s disease; acetylcholine; alternative splicing; hippocampus; VACHT

Significance Statement

In Alzheimer’s disease, degeneration of basal forebrain cholinergic neurons is an early event. These neurons communicate with target cells and regulate their long-term activity by poorly understood mechanisms. Recently, the splicing factor hnRNPA2/B, which is decreased in Alzheimer’s disease, was implicated as a potential mediator of long-term cholinergic regulation. Here, we demonstrate a mechanism by which cholinergic signaling controls the translation of hnRNPA2/B1 mRNA by activation of M1 muscarinic type receptors. Loss of cholinergic activity can have profound effects in target cells by modulating hnRNPA2/B1 levels.

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). Previous 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 sig-
naling (Dajas-Bailador and Wonnacott, 2004; Soreq, 2015). In addition, it has become clear that cholinergic signaling can also regulate long-term gene expression, miRNAs (Shaked et al., 2009; Soreq, 2015), and alternative splicing (Benson 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 (hnRNPA2/B1), which is reduced in brains of AD patients due to cholinergic deficiency (Benson et al., 2012; Kolisnyk et al., 2013a) and is reduced in mice with a conditional deletion of the vesicular acetylcholine (ACH) transporter in the forebrain (VACHT) (Kolisnyk et al., 2013a).

hnRNPs are a large family of proteins that package pre-mRNA into larger hnRNP particles (Dreyfuss et al., 1993). Each of the hnRNPs contains distinct RNA binding motifs, which allows them to exert their roles in pre-mRNA processing (Weighardt et al., 1996; Black, 2003). 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, knock-down of hnRNPA2/B1 in vivo caused impairments in learning and memory (Benson et al., 2012). 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 hnRNPA2/B1 translation. This work provides a new mechanism by which acetylcholine can influence targeted neurons, leading to potential widespread changes in neuronal function.

Materials and Methods

Mouse lines. All animals with targeted elimination of VACHT were generated using the VACHT<sup>flox/flox</sup> mouse described by Martins-Silva et al. (2011). To eliminate VACHT from the forebrain, VACHT<sup>flox/flox</sup> mice were crossed with C57BL/6-Tg(Nkx2–1-cre)2Sand/J mice (Xu et al., 2008) to generate VACHT<sup>flox<sub>Nkx2.1-Cre-flox</sub></sup> mice (Al-Onaizi et al., 2016). To eliminate VACHT from the striatum, the VACHT<sup>flox/flox</sup> mice were crossed with D2-Cre mice (Drd2, line E44) to generate VACHT<sup>flox<sub>D2-Cre-flox</sub></sup> (Guzman et al., 2011). VACHT-overexpressing ChAT-ChR2-EYFP mice were from The Jackson Laboratory (B6.Cg-Tg(Ch-Chop+H143R/EYFP)/Efggj; 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) was described previously. All procedures were conducted in accordance with guidelines of the Canadian Council of Animal Care at the University of Western Ontario with an approved institutional animal protocol (2008-127). Only male mice were used for all experiments.

Immunofluorescence. Immunofluorescence experiments were performed as described previously (Guzman et al., 2011). Primary antibodies used were anti-hnRNPA2/B1 (1:200; Santa Cruz Biotechnology, catalog sc-h-10035), anti-NeuN (1:200; sc-h-10035), anti-NeuN (1:200; sc-h-10035), anti-NeuN (1:200; sc-h-10035), anti-NeuN (1:200; sc-h-10035), anti-NeuN (1:200; sc-h-10035), anti-NeuN (1:200; sc-h-10035), anti-NeuN (1:200; sc-h-10035), anti-NeuN (1:200; sc-h-10035), and anti-NeuN (1:200; sc-h-10035). Sections were visualized with a Zeiss LSM 510 Meta confocal system (10 ×, 40 ×, and 63 × objectives), and a 488 nm Ar laser and 633 nm HeNe laser were used for excitation of fluorophores.

Western blotting. Mouse hippocampi were collected, proteins were isolated, and immunoblotting was performed as described previously using RIPA lysis buffer supplemented with protease and phosphatase inhibitors (Guzman et al., 2011). Band intensities were quantified using FluoroChemQ software (Thermo Fisher Scientific).

Subcellular fractionation. To isolate nuclear and cytoplasmic proteins from hippocampal tissue, the NE-PER Nuclear Protein Extraction Kit (Thermo Fisher Scientific) was used following the manufacturer’s instructions.

Sarkosyl insolvency. Isolation of sarkosyl-insoluble protein was performed as described previously (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% (w/v) 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% (w/v) sarkosyl (N-lauroylsarcosine)] for the sarkosyl-soluble fraction, and finally 8 ml urea with 2% (w/v) SDS for the sarkosyl-insoluble fraction.

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 Biotechnology (catalog sc-h-10035) antibody and run on an SDS-PAGE gel. The gel was then blotted with an anti-ubiquitin antibody (Abcam, catalog Ab7780).

Stereotaxic injections of adenovirus-associated virus. Injection of AAV virus was described previously (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 Gene Copies/ml) of AAV8-GFP-Cre or control virus (AAV8-GFP, Vector BioLabs) was injected into the medial septum (0.98 anteroposterior, 0.1 laterolateral, and 4.1 dorsoventral) of VACHT<sup>lox/lox</sup> mice. A recovery period of 4 weeks was given to allow transgene expression before subsequent analyses.

RNA sequencing. Total RNA was extracted from hippocampal tissues. The cDNA library was prepared using a TruSeq Stranded Total Sample Preparation kit (Illumina) and run in a HiSeq 2500 platform. Data sets are available on ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-3897.

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 described previously (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 described previously (Ribeiro et al., 2007).

Isolation of polysomal RNA. Isolation of polysomal RNA was performed as described previously (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 [VRCs]) supplemented with protease inhibitors (Complete, mini, EDTA-free, Roche). The homogenate was then centrifuged at 10,000 × g for 10 min at 4°C, and the supernatant was transferred to a fresh tube. The lysate was treated with either 30 mM EDTA or 1 mM RNase A for 30 min on ice; if it was to be treated with EDTA, VRCs were not included in the lysis buffer. Lysate was then carefully layered onto 15–55% of sucrose in 25 mM Tris-HCl, pH 7.4, 2.5 mM NaCl, 5 mM MgCl₂ (and 30 mM EDTA for the cDNA samples). Gradients were ultracentrifuged in a Beckman Instruments SW40Ti rotor at 150,000 × g for 2 h and 25 min at 4°C.

To isolate RNA from the fractions, RNA was collected into 1.5 ml of 7.5% guanidium-HCl, 2 ml of 100% ethanol was added, and samples were stored at −20°C. Samples were then centrifuged at 4000 × g for 50 min at 4°C. The supernatant was removed, and 200 µl DEPC-treated H₂O was added to the pellet. To precipitate the RNA, 300 µl of 100% ethanol, 20 µl of 3.0 M sodium acetate, pH 5.2, and 10 µg glycerol were added, and the samples were stored overnight at −20°C. The samples were then centrifuged at 14,000 × g for 30 min at 4°C. Pellets were washed with ice-cold 75% ethanol and air dried for 15 min at room temperature. RNA was resuspended 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.

Primary neuronal cultures. Primary cultures of hippocampal neurons from embryonic day 17 mouse embryos were obtained as described previously (Berardo 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 or 3 d. Neurons were cultured for 15 d.

Pharmacological manipulations in primary neuronal cultures. On the 15th day of culture, neurons were treated with different doses of carbamol (0.5, 10, or 50 µM) dissolved in saline, and 48 h 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 pretreated with mecamylamine (100 µM), atropine (100 µM), or both. Then, 1 h 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, or 100 µM) dissolved in saline.

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 were used.

Results

Cholinergic modulation of hnRNPA2/B1 protein levels

Previous experiments indicated that hnRNPA2/B1 is decreased in 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 hnRNPA2/B1 (Berson et al., 2012; Kolinsky et al., 2013a). As
expected, \( \text{VACht}^{\text{Nkx2.1-Cre-flox/flox}} \) animals presented a robust decrease in \( \text{hnRNPA2/B1} \) levels in the hippocampus (Fig. 1A).

The Nk2.1 promoter turns on Cre expression early during development (Xu et al., 2008), and, therefore, the resulting decrease in \( \text{hnRNPA2/B1} \) 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 VACHT\textsuperscript{Nkx2.1-Cre-flox/flox} mice using AAV8-Cre virus. AAV8-Cre-injected mice showed interrelated decline of both VACHT and hnRNPA2/B1 proteins, whereas AAV8-GFP-injected mice did not (Fig. 1B). There was a significant relationship between VACHT and hnRNPA2/B1 levels ($r^2 = 0.755$, $p < 0.001$; Fig. 1B). In addition, we tested whether, in the striatum, elimination of cholinergic tone would affect hnRNPA2/B1 expression by using VACHT\textsuperscript{D2-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. 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. 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 signaling in the CNS. To distinguish between these possibilities, we tested the hippocampus of TgR mice overexpressing 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. 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. 1F). 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 (Wolozin, 2012; Kim et al., 2013). We therefore determined by immunofluorescence staining whether the localization of hnRNPA2/B1 is changed in response to decreased cholinergic tone. Compared to controls, VACHT\textsuperscript{Nkx2.1-Cre-flox/flox} had a decrease in hnRNPA2/B1 immunostaining in the CA1, CA3, and dentate gyros regions of the hippocampus (Fig. 2A–D). hnRNPA2/B1 was present mainly in the nucleus of both neurons (labeled by NeuN; Fig. 2A–D) as well as in astrocytes (E) 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 whether VACHT\textsuperscript{Nkx2.1-Cre-flox/flox} mice show changes in hnRNPA2/B1 distribution between the nuclear and cytoplasmic fractions (Fig. 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, VACHT\textsuperscript{Nkx2.1-Cre-flox/flox} mice showed consistently reduced nuclear hnRNPA2/B1 levels (Fig. 2F).

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 deg-
radiation by the proteasome (Hochstrasser, 1996). Immuno-precipitated hnRNPA2/B1 from the hippocampi of controls and VAChT<sup>Nkx2.1-Cre-flox/flox</sup> mice was resolved by SDS-PAGE and probed with ubiquitin antibodies. VAChT-deficient mice showed no change in ubiquitination status of hnRNPA2/B1 and probed with ubiquitin antibodies. VAChT-deficient and three control mice in the absence or presence of EDTA. The numbers 1–12 are the fraction numbers. The values plotted are averaged from gels in C. Data shown are means.

We also examined whether cholinergic tone affects aggregation of hnRNPA2/B1. Notably, hnRNPA2/B1 has a prion-like domain that favors increased aggregation when mutated (Kim et al., 2013). Protein aggregation was investigated by fractionating hippocampal extracts into sarkosyl-soluble and sarkosyl-insoluble fractions (Fig. 3B). In both controls and VAChT-deficient mice, hnRNPA2/B1 was mainly present in soluble fractions, unlike the U1-70k small nuclear ribonucleoprotein, which has been shown to be present in insoluble fractions (Bai et al., 2013; Fig. 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. 3C), as observed in AD brains (Berson et al., 2012).

It was reported previously 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. 3D). We evaluated by qPCR the ratio of NMD-sensitive to NMD-insensitive versions of the hnRNPA2/B1 transcript in the hippocampus of VACHT<sup>Nkx2.1-Cre-flox/flox</sup> mice. Compared to controls, we observed a significant shift toward the NMD+ product in VAChT-deficient mice (Fig. 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. 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. 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. 4D, E). However, in VAChT<sup>Nkx2.1-Cre-flox/flox</sup> mice, distribution of hnRNPA2/B1 mRNAs was widespread throughout the fractions, resembling the distribution observed after EDTA treatment (Fig. 4D, E). The abundant β-actin mRNA remained unaltered between genotypes, demonstrating specificity toward hnRNPA2/B1 (Fig. 4F, 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.

**Muscarinic signaling regulates hnRNPA2/B1 translation by an NMD mechanism**

To further understand how cholinergic signaling regulates hnRNPA2/B1 levels, we treated neuronal hippocampal cultures from wild-type mice with the cholinergic mimetic carbachol (10 μM; Fig. 5A, 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. 5C).

To study the contribution of muscarinic receptor subtypes, we evaluated hnRNPA2/B1 levels in the hippocampus of muscarinic receptor knock-out mice. Compared to wild-type mice, M1, but not M4, receptor knock-out mice showed a decrease in hnRNPA2/B1 protein levels resembling that in VACHT<sup>Nkx2.1-Cre-flox/flox</sup> mice.
mice (Fig. 5D). Together, these experiments suggest that decreased cholinergic tone, likely due to insufficient M1 receptor activation, changes hnRNP A2/B1 protein levels by regulating mRNA translation.

To further investigate the importance of M1 muscarinic receptors in the regulation of hnRNP A2/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 hnRNP A2/B1 (Fig. 5E).

We then evaluated whether in vitro cholinergic regulation also changes the NMD + product. Compared to saline treatment, carbachol shifted the expression of hnRNP A2/B1 RNA toward the NMD insensitive full-length mRNA product (Fig. 5F). Furthermore, cotreatment with atropine blocked this effect and returned the ratio to control levels, similar to what we observed for hnRNP A2/B1 protein levels (Fig. 5C). These data suggest that the ratio of NMD + hnRNP A2/B1 gene products predicts change in protein levels, and that the regulation of NMD sensitivity is dependent on muscarinic signaling.

Discussion

In this study, we combined a variety of in vivo and in vitro techniques to evaluate the contribution of cholinergic signaling to expression levels of hippocampal hnRNP A2/B1 protein. Using a number of mouse lines, we demonstrated that hnRNP A2/B1 protein levels in the hippocampus are exquisitely sensitive to changes in cholinergic tone.

Interestingly, we observed no change in hnRNP A2/B1 protein levels in striatum-specific VACH T mutants, whereas there is a body of evidence that cholinergic signaling can affect the levels of this protein in both cortical and hippocampal regions in vivo (Berson et al., 2012; Kolinsky 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 hnRNP A2/B1 translation in response to cholinergic activity. Our results highlight the critical role of the Gq coupled M1 muscarinic receptor in governing hnRNP A2/B1 protein levels.

Unlike the rare hnRNP A2/B1 mutation that increases aggregation and nuclear exclusion (Kim et al., 2013), we did not find aggregation of hnRNP A2/B1 in mice with forebrain cholinergic deficiency, suggesting that in these mice, and likely in AD brains, hnRNP A2/B1 dysfunction occurs by a separate and distinct mechanism. Furthermore, we did not see an increase in ubiquitination, suggesting that changes in hnRNP A2/B1 protein levels do not occur at the posttranslational level. In line with this, we found that it may in fact be abnormal translation that drives regulation of hnRNP A2/B1.

A common mechanism for the regulation of the translation of RNA binding proteins (RBPs) is regulation by unproductive splicing and translation (RUST), where the alternative splicing 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 hnRNP A2/B1 transcripts were maintained in cholinergic-deficient mice or AD brains (Berson et al., 2012), but hnRNP A2/B1 translation was selectively decreased. Notably, hnRNP A2/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 mTORC (McGlincy et al., 2010; Dempsey, 2012), which is a key effector of muscarinic receptor signaling (Slack and Blusztajn, 2008). Correspondingly, we found that cholinergic control of hnRNP A2/B1 translation is mediated by M1 muscarinic receptors (Fig. 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 (Tollery et al., 2011; Berson et al., 2012; Bai et al., 2013; Qian and Liu, 2014). A number of substances, including regularly prescribed antibiotics, have been shown to nonselectively alter alternative splicing in the brain (Graveley, 2005; Tollery et al., 2011; Kole et al., 2012); 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 Alzheimer’s Disease Neuroimaging Initiative 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 signaling to the etiology of AD comes from clinical evidence that the long-term use of anticholinergic medication, specifically antimuscarinic drugs, significantly increases the risk of developing dementia (Gray et al., 2015). Interestingly, administration of antimuscarinic 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 signaling.

The main pathological hallmarks of AD are the accumulation of Aβ plaques and of hyperphosphorylated tau (Huang and Jiang, 2009). M1 muscarinic signaling 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 hnRNP A2/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 VACH T have severe deficits in performance on the paired-associates learning touch-screen task (Al-Onaizi et al., 2016), a rodent version of the Cambridge Neuropsychological Test Automated Battery tests used in humans, which has been
shown to be selective for the memory impairments in AD patients (Egerhazi et al., 2007). Importantly, lenti- viral mediated knockdown of hnrNP A2/B1 also produced cognition impairments in mice (Berson et al., 2012).

Together, our findings indicate an intricate relationship between M1 muscarinic signaling and hnrNP A2/B1 translation. These findings lay the ground work for new therapeutic avenues for the treatment of AD. Specifically, they point 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 nonhuman primates (Lange et al., 2015).

References


Appendix D- α7 nicotinic ACh receptor-deficient mice exhibit sustained attention impairments that are reversed by β2 nicotinic ACh receptor activation.

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**Other Terms and Conditions:**
RESEARCH PAPER

α7 nicotinic ACh receptor-deficient mice exhibit sustained attention impairments that are reversed by β2 nicotinic ACh receptor activation

Benjamin Kolisnyk1,2, Mohammed A. Al-Onaizi1,4, Vania F. Prado1,2,3,4 and Marco A. M. Prado1,2,3,4

BACKGROUND AND PURPOSE
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.

EXPERIMENTAL APPROACH
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.

KEY RESULTS
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.

CONCLUSIONS AND IMPLICATIONS
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.

Abbreviations
5-CSRT, five-choice serial reaction time task; VAChT, vesicular ACh transporter
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; Guillemot-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; Guillemot-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 a7nAChR-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 may be of potential use for correcting cognitive and molecular signalling deficits seen in psychiatric or neurological disorders in which α7nAChRs are affected.

Methods

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 (07:00–19:00h), 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).

**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 (Kolinsky et al., 2013a, b).

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.

**Probe trial**
To probe attention in mice, we used a previously described probe trial schedule with reduced stimulus durations (Romberg et al., 2011; Kolinsky 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.

**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) experiment (B Crisis et al., 2013); mice 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 (AChT) (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).

**Statistical analyses**
All data are expressed as mean ± SEM. SIGMA STAT 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.

**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.

**Quantitative PCR**
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; Kolinsky et al., 2013a).

**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-β-actin (catalogue #ab49900; Abcam, Cambridge, UK). Band intensity was quantified using FLUOROCHEMQ software (Thermo Fisher Scientific).
Results

a7nAChR-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 [Supporting Information Fig. 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, \(F_{(1,14)}=4.472, P=0.0529\); Supporting Information Fig. 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).

a7nAChR-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 1A] 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 1B]. 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 1C] 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 1D]. 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 1E]. 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 1F].

Figure 1

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).
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 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 2A]. 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 2B] 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 2C]. 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 2D] 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 2E] 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 2F] 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 2G]. 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, $F_{(3,36)}=0.09521, P=0.9621$; Supporting Information Fig. 2C, D], 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, $F_{(3,36)}=0.8660, P=0.4685$, Supporting Information Fig. 2C, D].

The ability to release normal levels of ACh is critical to attention (Kolinsyk 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 $0.375, P=0.7291$] or ChAβ. The sustained attention deficits in CHRNA7/−/− mice are therefore not a result of an inherent dysfunction in the machinery required for ACh release (Figure 3A).

Figure 2

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).

and observed no significant difference between genotypes [t(8) = 0.3016, P = 0.7677]. In addition, we evaluated the expression of the enzyme AChe and observed no significant difference between genotypes [t(8) = 0.0409, P = 0.9864]. As expected, we did not detect CHRNA7 expression in CHRNA7−/− animals (Figure 3B).

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 3C]. 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 3D].

**Effect of a7nAChR agonists on attention**

To evaluate acute roles of a7nAChR in regulating sustained attention behaviour, we investigated two selective a7nAChR 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 omisions [RM-ANOVA: main effect of dose, F(3,18) = 12.52, P < 0.001; Figure 4A], 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 4B]. 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, Supporting Information Fig. 3A] or reward collection latency [RM-ANOVA: no effect of dose, F(3,18) = 0.7517, P = 0.5382; Supporting Information Fig. 3B]. In addition, PHA-543,613 did not alter premature [RM-ANOVA: no effect of dose, F(3,18) = 0.7999, P = 0.4930] nor perseverative responses [RM-ANOVA: no effect of dose, F(3,18) = 1.404, P = 0.8821, Supporting Information Fig. 3C, 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 4C], 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) = 0.0437; Figure 4D]. 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 a7nAChR agonist tested, PNU-282,987, also significantly improved rate of omissions [RM-ANOVA: main effect of dose, F(3,36) = 2.767, P = 0.0437, Figure 4E], with post hoc analysis confirming that both the 1 and 3 mg kg−1 doses significantly improved performance over saline. Conversely, PNU-282,987 significantly altered response accuracy [RM-ANOVA: main effect of dose, F(3,18) = 5.637, P = 0.0066; Figure 4F]. Post hoc analysis revealed that at the highest dose tested (5 mg kg−1) mice injected with PNU-282,987 performed significantly worse than mice injected with saline. PNU-282,987 did not significantly alter response latency [RM-ANOVA: no effect of dose, F(3,36) = 0.9985, P = 0.4018; Supporting Information Fig. 3E] nor reward collection latency [RM-ANOVA: no effect of dose, F(3,18) = 1.131, P = 0.3375; Supporting Information Fig. 3F]. PNU-282,987 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; Supporting Information Fig. 3G, H]. In terms of the effects of PNU-282,987 on sustained attention, analysis of injected mice over two blocks of 25 trials showed that PNU-282,987 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 4G], but did not alter sustained accuracy [RM-ANOVA: main effect of dose, F(3,36) = 1.692, P = 0.1852].

**Figure 3**

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).

a7 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 CHRRB2 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 3B).
Figure 4

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).

dose, F(3,56) = 6.044, P = 0.0019; no effect of block, F(3,12) = 0.222, P = 0.6458; and no interaction effect, F(3,18) = 0.0701, P = 0.9755; Figure 4I].

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 4J], 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 4J].

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 S-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 5A] or response accuracy [RM-ANOVA: no effect of dose, F(3,18) = 0.1121, P = 0.8733; Figure 5B], response latency [RM-ANOVA: no effect of dose, F(3,18) = 0.2490, P = 0.7348; Supporting Information Fig. 4A] or reward collection latency [RM-ANOVA: no effect of dose, F(3,18) = 0.3018, P = 0.6621; Supporting Information Fig. 4B]. 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, Supporting Information Fig. 4C, 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(3,18) = 1.528, P = 0.2414; main effect of block, F(3,12) = 14.89, P = 0.0084; and no interaction F(3,12) = 0.2209, P = 0.8806; Figure 5C]. PHA-543,613 did not alter sustained accuracy in CHRNA7−/− mice either [RM-ANOVA: no effect of dose, F(3,30) = 0.2017, P = 0.8945; no effect of block, F(3,12) = 0.00701, P = 0.9343; and no interaction, F(3,30) = 0.02177, P = 0.9956; Figure 5D].

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 5E], response accuracy [RM-ANOVA: no effect of dose, F(3,18) = 0.1458, P = 0.9310; Figure 5F], response latency [RM-ANOVA: no effect of dose, F(3,18) = 0.0586, P = 2.808, Supporting Information Fig. 4E] or reward collection latency [RM-ANOVA: no effect of dose, F(3,18) = 0.8089, P = 0.4603, Supporting Information Fig. 4F]. PNU-282,987 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, Supporting Information Fig. 4G, 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(1,16) = 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 5G] nor on sustained accuracy [RM-ANOVA: no effect of blocks,
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).

β2nAChR agonists improve 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 6A] and response accuracy [RM-ANOVA: main effect of dose, F(3,18) = 6.950, P = 0.0020; Figure 6B] without altering response latency [RM-ANOVA: no effect of dose, F(3,18) = 0.06377, P = 0.9014; Supporting Information Fig. 5A] or reward collection latency [RM-ANOVA: no effect of dose, F(3,18) = 0.2936, P = 0.8797; Supporting Information Fig. 5B]. 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, Supporting Information Fig. 5C, 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 6C].
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 6D].

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 7A] and accuracy [RM-ANOVA: main effect of dose, $F_{(3,18)}=3.383, P=0.0373$; Figure 7B] in CHRNA7−/− mice, without altering response latency [RM-ANOVA: no effect of dose, $F_{(3,18)}=1.622, P=0.2495$; Supporting Information Fig. 6A] or reward collection latency [RM-ANOVA: no effect of dose, $F_{(3,18)}=0.8793, P=0.4359$; Supporting Information Fig. 6B]. ABT-418 did not alter the number of premature [RM-ANOVA: no effect of dose, $F_{(3,18)}=0.1450, P=0.8113$] or perseverative responses [RM-ANOVA: no effect of dose, $F_{(3,18)}=0.1254, P=0.8336$; Supporting Information Fig. 6C, D]. Importantly, ABT-418 was able to reverse the sustained attention deficits observed in CHRNA7−/− mice (Figure 7C) 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 7C]. 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 7D].

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 7E]. 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 7F]. 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 [one-way ANOVA: no effect of treatment, $F_{(6,18)}=0.7467, P=0.4692$; Supporting Information Fig. 7A] and accuracy [one-way ANOVA: no effect of treatment, $F_{(6,18)}=0.6749, P=0.6716$; Supporting Information Fig. 7B] and CHRNA7−/− mice [omission [one-way ANOVA: no effect of treatment, $F_{(6,18)}=2.565, P=0.1154$; Supporting Information Fig. 7C] and accuracy [one-way ANOVA: no effect of treatment, $F_{(6,18)}=1.005, P=0.3876$; Supporting Information Fig. 7D]] demonstrated no significant change in performance in both omissions and accuracy from their naive performance across all injections.

**Figure 7**
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.
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 expression of VAcChT and increased cholinergic signalling in BAC ChAcChr2-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 (Gioannini 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 methyllycarnitine, 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).

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Author contributions

B.K. Designed, performed experiments, analyzed data and wrote the manuscript, MAO performed experiments, VFP and MAMP conceived experiments and wrote paper.

Conflict of interest

The authors declare no competing financial interests.

References


Nicotinic receptors and sustained attention


Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:
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Figure S1 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 S2 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 S3 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-S43,613 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 S4 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-S43,613 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 S5 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 S6 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 S7 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.)
Appendix E- Forebrain deletion of the vesicular acetylcholine transporter results in deficits in executive function, metabolic, and RNA splicing abnormalities in the prefrontal cortex.

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Contributions to publication: M.A.A-O assisted in testing VACht^{Six3-Cre-flox/flox} mice on pairwise visual discrimination touchscreen task.
Forebrain Deletion of the Vesicular Acetylcholine Transporter Results in Deficits in Executive Function, Metabolic, and RNA Splicing Abnormalities in the Prefrontal Cortex

Benjamin Kolisnyk,1,2 Mohammed A. Al-Onaizi,1,4 Pedro H. F. Hirata,1 Monica S. Guzman,1,3 Simona Nikolova,1 Shahar Barbash,3 Hermona Soreq,6 Robert Bartha,1,2,3 Marco A. M. Prado,1,2,3,4 and Vania F. Prado1,2,3,4

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.

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; Kolinsky 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 (Éfangé 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.

Materials and Methods

Animals. Generation of VACHT\textsuperscript{Six3-Cre-flox/splox} mice was previously described (Martyn et al., 2012). In short, VACHT\textsuperscript{Six3-Cre-flox/splox} mice were generated by crossing VACHT\textsuperscript{flox/flox} (mixed C57BL/6f \times 129/SvEv background, backcrossed to C57BL/6f for five generations) with the Six3-Cre mouse line (NMRI background, backcrossed to C57BL/6f for five generations). We then intercrossed VACHT\textsuperscript{Six3-Cre-flox/splox} mice to obtain VACHT\textsuperscript{Six3-Cre-flox/splox}. For the galantamine experiments, the mice used were wild-type C57BL/6f. 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 \textit{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).

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-Syntrophin (catalog #55768; Sigma-Aldrich) at a 1:500 dilution, anti-\textit{h}-rNAP A2/B1 (catalog #sc-10035; Santa Cruz Biotechnology) at a 1:500 dilution, and anti-\textit{b}-Actin (catalog #aB49900; Abcam) at a 1:15000 dilution. Band intensity was quantified using FluoroChemQ software (Thermo Fisher Scientific).

ACh release. ACh release from prefrontal cortical brain slices was quantified by labeling slices with \textit{[3]H} methyl-choline before using KCl to stimulate release of labeled \textit{ACH} as previously described (Guzman et al., 2011).

\textit{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 \textit{qPCR} analysis were performed as previously described (Guzman et al., 2011). Primer sequences used to determine alternative spliced transcripts are found in Table 1. \textit{\textit{b}}-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.

\textit{Magnetic resonance imaging}. Magnetic resonance spectroscopy of the prefrontal region was performed in four VACHT\textsuperscript{Six3-Cre-flox/splox} mice and four littersmates (VACHT\textsuperscript{flox/flox}). Spectroscopic localization of a 24 μl voxel was achieved by adiabatic selective refocusing (DelaBarre and Garrett, 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). 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).

\textit{Touchscreen behavioral assessment}

\textit{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--Sakida Mouse Touchscreen Systems model 81426 (Campden Instruments). Schedules were designed and data were collected using the ABET II Touch software v.2.13 (Lafayette Instruments).

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 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 received the reward a new trial was initiated. This

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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.

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.

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,
reward tone, was played semirandomly 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.

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.

Drug treatment. 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.

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.

Results VACHT\textsuperscript{Six3-Cre-flox/lox} mice have reduced VACHT and ACh release in the PFC

We have previously reported that VACHT\textsuperscript{Six3-Cre-flox/lox} 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 per-
formed Western blot analysis to assess VACHT expression. VACHT<sup>Six3-Cre-flox/flox</sup> mice have a significant reduction in PFC VACHT protein expression (t<sub>6</sub> = 2.706, p = 0.0353; Fig. 1A). Moreover, this reduction in VACHT protein levels results in a significant decrease in newly synthesized [<sup>3</sup>H] ACh release in slices of PFC from VACHT<sup>Six3-Cre-flox/flox</sup> mice when compared with control mice (t<sub>6</sub> = 2.899, p = 0.0442; Fig. 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 VACHT<sup>Six3-Cre-flox/flox</sup> mice show no significant change in the expression of α7nAChR mRNA (t<sub>10</sub> = 0.9300, p = 0.3743) nor β2nAChR mRNA (t<sub>10</sub> = 0.8359, p = 0.4227). Interestingly, α4nAChR mRNA is slightly upregulated (t<sub>10</sub> = 2.550, p = 0.0289; Fig. 1C).

Decreased forebrain cholinergic tone specifically disturbs reversal learning in the pairwise visual discrimination task. We have previously demonstrated that VACHT<sup>Six3-Cre-flox/flox</sup> 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 VACHT<sup>Six3-Cre-flox/flox</sup> 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 VACHT<sup>Six3-Cre-flox/flox</sup> mice did not differ from that of controls (VACHT<sup>+/+</sup>) 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<sub>(1,54)</sub> = 0.3950, p = 0.5398; main effect of training phase F<sub>(4,54)</sub> = 5.227, p = 0.0012; and no interaction effect F<sub>(4,54)</sub> = 1.389, p = 0.2495; Figure 2A). Acquisition of the pairwise visual discrimination task (Fig. 2B; for stimuli used) did not differ between genotypes, in terms of sessions to criteria (Fig. 2C; t(14) = 0.2446, p = 0.8117), correction errors made (Fig. 2D; t(14) = 0.2942, p = 0.7746), response latency (Fig. 2E; t(14) = 1.019, p = 0.3256), or reward collection latency (Fig. 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, VACHT<sup>Six3-Cre-flox/flox</sup> mice showed severe reversal learning impairment (Fig. 3A; for the stimuli used), measured by the percentage of correct responses (Fig. 3B; RM-ANOVA, main effect of genotype F<sub>(1,132)</sub> = 19.78, p = 0.0008; main effect of session F<sub>(11,132)</sub> = 23.28, p = 0.0001; and significant interaction effect F<sub>(1,12)</sub> = 5.035, p = 0.0001) and by its increased correction errors (Fig. 3C; RM-ANOVA, main effect of genotype F<sub>(1,132)</sub> = 14.72, p = 0.0024; main effect of session F<sub>(11,132)</sub> = 14.37, p = 0.0001; and significant interaction effect F<sub>(1,12)</sub> = 2.817, p = 0.0025). Post hoc analysis showed that VACHT<sup>Six3-Cre-flox/flox</sup> 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 VACHT<sup>Six3-Cre-flox/flox</sup> mice did not differ from controls in terms of response latency (Fig. 3D; RM-ANOVA, no effect of genotype F<sub>(1,154)</sub> = 0.4233, p = 0.5258; main effect of session F<sub>(11,154)</sub> = 4.705, p = 0.0001; and no interaction effect F<sub>(11,154)</sub> = 0.9997, p = 0.4493) or reward collection latency (Fig. 3E; RM-ANOVA, no effect of genotype F<sub>(1,154)</sub> = 1.107, p = 0.3105; main effect of session F<sub>(11,132)</sub> = 3.965, p = 0.0001; and no interaction effect F<sub>(11,132)</sub> = 3.965, p = 0.0001). These results suggest that forebrain cholinergic tone is important for cognitive flexibility.

VACHT<sup>Six3-Cre-flox/flox</sup> mice have impaired acquisition of the 5-CSRT task

Similar to the results for visual discrimination experiments, the performance of the second cohort of VACHT<sup>Six3-Cre-flox/flox</sup> 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<sub>(1,32)</sub> = 1.528, p = 0.2515; no effect of training phase F<sub>(4,32)</sub> = 1.492, p = 0.2280; Figure 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), VACHT<sup>Six3-Cre-flox/flox</sup> showed a significantly worse performance, needing more sessions to reach criteria (RM-ANOVA, main effect of genotype, F<sub>(1,8)</sub> = 10.06, p = 0.0132; main effect of stimulus duration, F<sub>(1,8)</sub> = 5.731, p = 0.0436, no interaction effect, F<sub>(1,8)</sub> = 4.252, p = 0.0731; Fig. 4B).

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, VACHT<sup>Six3-Cre-flox/flox</sup> mice were able to perform identically to control mice in both accuracy and omisions (Fig. 5A,B). In contrast, increasing attentional demand by diminishing the stimulus period revealed an attentional deficit in VACHT<sup>Six3-Cre-flox/flox</sup> mice. Response accuracy did not differ between genotypes (RM-ANOVA, no effect of genotype F<sub>(1,24)</sub> = 0.0007548, p = 0.9788; main effect of stimulus duration F<sub>(3,24)</sub> = 10.28, p < 0.001; Figure 5A). However, VACHT<sup>Six3-Cre-flox/flox</sup> mice presented an increased rate of omission (RM-ANOVA, main effect of genotype F<sub>(1,24)</sub> = 57.99, p < 0.0001; main effect of stimulus duration...
Post hoc analysis revealed that VAChT\textsuperscript{Six3-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 $F_{(3,24)} = 0.07440$, $p = 0.7874$; no effect of stimulus duration $F_{(3,24)} = 0.9891$, $p = 0.4146$; Figure 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 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 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 5F).

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 6A). In contrast, response accuracy of VAChT\textsuperscript{Six3-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 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 $F_{(3,48)} = 5.803$, $p = 0.0104$; no effect of block $F_{(4,48)} = 0.5352$, $p = 0.7105$; Figure 6C), whereas rate of omissions of VAChT\textsuperscript{Six3-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 6D). Together the data suggest that VACHT$\text{Six3-Cre-flox/flox}$ mice have impaired ability to sustain attention.

**Deletion of forebrain VACHT increases susceptibility to distractions**

To assess distractibility of VACHT$\text{Six3-Cre-flox/flox}$ 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 VACHT$\text{Six3-Cre-flox/flox}$ 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 7A). Rate of omission was significantly higher in VACHT$\text{Six3-Cre-flox/flox}$ 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 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 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 7D).

**Galantamine improves attention in wild-type mice on a demanding task**

Detection of pre-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. 8A) and rate of omissions ($F(4,31) = 6.716, p = 0.0024$; Fig. 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. 8C) or omissions (paired $t$ test, $t_{(7)} = 0.4581, p = 0.6661$; Fig. 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. 8E) and significantly reduced the rate of omission (paired $t$ test, $t_{(7)} = 2.379, p = 0.0489$; Fig. 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.

**Galantamine does not improve attention deficits in VACHT$\text{Six3-Cre-flox/flox}$ mice**

To investigate if the deficits observed in VACHT$\text{Six3-Cre-flox/flox}$ 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. 8), and tested both VACHT$\text{Six3-Cre-flox/flox}$ and VACHT$\text{Six3-Cre-flox/flox}$ 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. 8G). However, the drug was able to significantly reduce rate of omissions (paired $t$ test, $t_{(5)} = 3.383, p = 0.0277$; Fig. 8H) in VACHT$\text{Six3-Cre-flox/flox}$ mice. Interestingly, galantamine had no effect on the performance of VACHT$\text{Six3-Cre-flox/flox}$ 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. 8H).

**Deletion of forebrain VACHT results in metabolic abnormalities in the PFC**

The lack of effect of galantamine in VACHT$\text{Six3-Cre-flox/flox}$ 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. 9A; representative spectra).
This analysis revealed that VAChT^{Six3-Cre-flox/flox} mice had significantly less Lac ($t_{(6)} = 2.600, p = 0.0428$; Fig. 9B) and Tau ($t_{(6)} = 2.522, p = 0.0452$; Fig. 9C) than controls. Levels of NAA ($t_{(6)} = 0.9097, p = 0.9307$; Fig. 9D), Myo ($t_{(6)} = 0.9598, p = 0.3742$; Fig. 9E), and Cho ($t_{(6)} = 0.1461, p = 0.8886$; Fig. 9F) remained unchanged.

**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 VAChT^{Six3-Cre-flox/flox} shows parallel suppression of the hnRNPA2/B1 proteins, their levels were assessed. Compared with controls, the PFC of VAChT^{Six3-Cre-flox/flox} mice showed a significant reduction in hnRNPA2/B1 expression (75% decrease, $t_{(6)} = 4.941, p = 0.0026$; Fig. 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, SIPAIL1(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 VAChT^{Six3-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. 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 VAChT^{Six3-Cre-flox/flox} mice, we performed qPCR to determine the expression of the PKM1 and PKM2 splice variants. Interestingly the VAChT^{Six3-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. 10C), effectively changing the ratio between these two enzymes.

Figure 8. Galantamine improves attention in wild-type, but not in VAChT^{Six3-Cre-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 (1 mg/kg i.p.) 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^{Six3-Cre-flox/flox} and VAChT^{Six3-Cre-flox/flox} mice ($n = 6$; data are mean ± SEM, *p < 0.05).
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.

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 (Balchun 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.

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 affected. 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 improv-
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.

**Figure 10.** VAChT<sup>Six3-Cre-flox/flox</sup> mice have abnormal RNA processing in the PFC. **A**, hnRNP A2/B1 protein expression in the PFC with representative immunoblots (inset, n = 4). **B**, qPCR analysis of alternative splicing events for CD55, ENA, 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).
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.

The reduction in PFC hnRNPA2/B1 observed in VACHT-Six3-Cre-flox/Box 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 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 VACHT-deficient mice may be useful for understanding metabolic abnormalities that occur in dementia.

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 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 (Efan 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.

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Curriculum Vitae

Full Name: Mohammed A. Al-Onaizi

Education

- 2012-Present
  The University of Western Ontario
  London, ON
  PhD student in the Department of Anatomy and Cell Biology- Robarts Research Institute.

- 2010-2012
  The University of Dundee
  Dundee, UK
  Graduated with a Master’s (M.Sc.) degree in Human Anatomy- The Centre for Anatomy and Human Identification.

2006-2012
Kuwait University
Kuwait City, KW
Graduated with a Bachelor’s (B.Sc.) degree in Physiotherapy- Faculty of Allied Health.

Employment

Sept. 2012 – Present
Ph.D. Fellowship- Department of Anatomy (Kuwait University)

PROFESSIONAL AFFILIATIONS

Society for Neuroscience (SfN)
Southern Ontario Neuroscience Association (SONA)

Awards and Scholarships

- Ph.D. Fellowship- Kuwait University (September 2012-August 2016)
- January 2016-Publication Award- Kuwait University.
- September 2014- Travel Award- Kuwait University.
- September 2014- London Health Research Day 2015- Student Choice Award
Abstracts and Presentations

- Cholinergic regulation of hippocampal-dependent information processing and Alzheimer’s-like pathology.

- Cholinergic regulation of hippocampal-dependent information processing, neurogenesis, and Alzheimer’s-like pathology.

- Cholinergic regulation of hippocampal-dependent information processing: implications for Alzheimer’s disease.

March 2015. Poster Presentation- Alzheimer’s and Parkinson’s Diseases Conference (Nice, France)
- Cholinergic regulation of hippocampal-dependent information processing: implications for Alzheimer’s disease.

- Deficits in cognitive function in a novel mouse model of basal forebrain cholinergic dysfunction

May 2014. Poster Presentation- Southern Ontario Neuroscience Association (London, CA)
- Deficits in cognitive function and disruption in hippocampal neurogenesis due to long-term deficient cholinergic signaling.

January 2014. Oral Presentation- Department of Anatomy at Kuwait University (Kuwait City, KW)
- Regulation of cognitive processing by forebrain and hippocampal cholinergic tone.

- Molecular mechanisms involved in cholinergic control of hippocampal-dependent learning and memory.

- Deficits in cognitive function, disruption in hippocampal neurogenesis and *de novo* plaque formation due to long-term decrease in cholinergic function.

**Related Work Experience:**
Teaching Assistant
The University of Western Ontario
2015-2016

**Publications:**


Kolisnyk B, Al-Onaizi MA, Prado VF, Prado MA. 2015. alpha7 nicotinic ACh receptor-deficient mice exhibit sustained attention impairments that are reversed by beta2 nicotinic ACh receptor activation. British journal of pharmacology 172:4919-4931.


*Equal contribution