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Cholinergic Modulation of Synaptic Plasticity in VAChT-Modified Mice

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By

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Abstract:

The hippocampus is a region of the brain known for its role in learning and memory. The neural correlate of memory formation is believed to be changes in synaptic efficacy through processes broadly termed "synaptic plasticity". Synaptic plasticity includes long term changes that increases (potentiation, LTP) or decreases (depression) synaptic strength. The hippocampus receives modulatory cholinergic afferents originating in the basal forebrain. This project investigates changes in bidirectional synaptic plasticity in gene-modified vesicular acetylcholine transporter protein-knockdown (VAChT-KD) mice, which express decreased acetylcholine secretion. Extracellular field recordings were performed on hippocampal slices to characterize synaptic physiology in the CA1-region glutamatergic synapses. VAChT-KD mice expressed reduced basal transmission and activity-dependent LTP, though depression was not changed. Furthermore, acute application of the cholinergic agonist carbachol rescued the LTP deficit. This project demonstrates that decreased cholinergic tone can affect hippocampal synaptic processes and suggests mechanisms by which cholinergic pathways may act on synapse physiology.

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List of Abbreviations:

α7R – nicotinic α7 receptor

ABP – AMPAR binding protein

ACh – acetylcholine

AChE - acetylcholinesterase

AKAP – A kinase-anchoring protein

AMPAR - α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor

BCM – Bienenstock-Cooper-Munro

CAMKII - Ca^{2+}/calmodulin-dependent protein kinase II

CCh – carbachol

ChAT – choline acetyltransferase

ChT – choline transporter

DAG - diaglycerol

EPSP – excitatoty postsynaptic potential

ERK – extracellular-signalling kinases

fEPSP – field EPSP

GPCR – G protein-coupled receptor

GRIP – glutmate receptor interacting protein

HDB – horizontal diagonal band of Broca

HFS – high frequency stimulation

IP3 – inositol triphosphate

LFS – low frequency stimulation

LTD - Long-term depression

LTP – long-term potentiation

mAChR – muscarinic receptor

MAPK - Mitogen-activated protein kinase

MBN – magnocellular basal nucleus

mGluR – metabotropic glutamate receptor

mLTP – muscarinic LTP

MS – medial septum

nAChR – nicotinic acetylcholine receptor

NMDAR - N-methyl-D-aspartic acid receptor

NR2A-R – NMDAR with two NR2A subunits

NR2B-R – NMDAR with two NR2B subunits

PACAP – pituitary adenylate cyclase activating peptide

PICK1 – Protein Interacting with C-kinase 1

PIP2 - Phosphatidylinositol 4,5-bisphosphate

PLC – phospholipase C

PP1 – Protein Phosphatase 1

PPF – paired-pulse faciliation

PSD – postsynaptic density

SFK – Src family kinase

STP – short term potentiation

VAChT – vesicular acetylcholine transporter

VAChT-KD – VAChT knocked down (mutation)

VDB – vertical diagonal band of Broca

VDCC – voltage-dependent calcium channel

INTRODUCTION

1. BACKGROUND
2. RATIONALE/HYPOTHESIS

In the last 40 years, there has been a great deal of scientific investigation into the biophysical and molecular basis of memory [1, 2]. The central theory is that information is physically stored as synaptic connections in the nervous system and that learning is obtained through the strengthening of specific synapses and depression of others through processes broadly termed "synaptic plasticity" [3, 4]. There is increasing evidence that acetylcholine (ACh) originating from basal forebrain neurons modulates the neural mechanisms involved in synaptic plasticity [5-7]. Cholinergic neurons originating in the basal forebrain project to several cortical and subcortical areas including the hippocampus [8], a brain region considered to have a functional role in memory [9, 10]. This project uses recently generated gene-modified mice which exhibit altered cholinergic tone to study cholinergic modulation of synaptic plasticity.

1. Background

1.1 The Hippocampus

1.1.1 Role in Cognition and Memory

The hippocampus plays a central role in memory processes and its physiological function in cognition and memory has been extensively researched for several decades [11]. Perhaps the most famous example of hippocampal function is in an epileptic patient known to the scientific community as H.M. who, in the 1950s, was surgically treated with bilateral excision of the medial temporal lobes. Though the procedure was successful in controlling his epilepsy, the patient suffered from severe anterograde amnesia specifically affecting declarative memory[12].

Early scientific studies with small mammals used lesioning and in vivo recording techniques to understand hippocampal function. Anatomically, the hippocampus is a

limbocortical structure consisting of two bilateral horns lining the medial temporal lobes[13, 14]. Hippocampal lesion studies revealed an array of behavioural alterations in rodents and opened the door for further investigation into the possible roles the hippocampus might play, which included motivation, behavioural inhibition, and various forms of learning and memory consolidation [15]. Particularly interesting single-unit recordings included the discovery of increased hippocampal activation in rabbits during associative learning [16] and the discovery of place cells [17], which increased their firing rate depending on spatial location and orientation. The latter finding led to determining the hippocampus' role in spatial memory and context-based learning. In 1982, Morris et al. demonstrated that rats with hippocampal lesions present impaired performance on spatial-memory tasks [10].

1.1.2 Neurocircuitry

Transverse sectioning of the hippocampus reveals a simple, unidirectional, laminar circuitry with a single layer of pyramidal neuron cell bodies lining the middle layer (the *stratum pyramidale*) . These pyramidal neurons are oriented so that their basal dendrites occupy the outer layer (*stratum oriens*) and apical dendrites occupy the inner layers (*stratum radiatum* and *stratum lacunosum/moleculare*). Cortical input into the hippocampus is principally via the entorhinal cortex and projects mainly to the dentate gyrus granule cells. Granule cells project their mossy fibres to the CA3 pyramidal neurons, which then project glutamatergic fibres to CA1 pyramidal cells via axons along the stratum radiatum known as "Schaffer collaterals" (Figure 1.1). CA1 neurons then project out of the hippocampus to cortical and other limbic regions such as the amygdale [9]. In addition to the principal network described above, hippocampal networks are regulated by GABAergic interneurons as well as extrinsic afferents such as cholinergic fibres from the basal forebrain [18, 19].

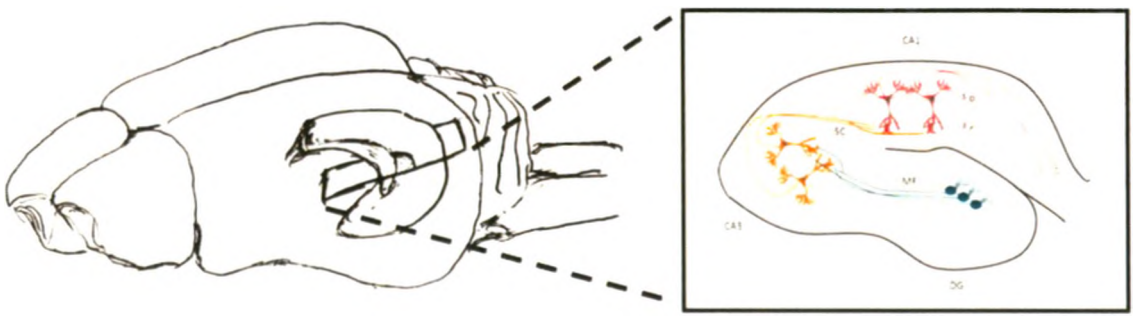


Figure 1.1. Structure and location of hippocampal horn within the medial temporal lobe. *Inset*, Transverse slice of hippocampus illustrating the principal trisynaptic circuitry. Somas of pyramidal cells are located along the stratum pyramidale (grey line). Basal dendrites are present in the stratum oriens (s.o.) and apical dendrites are situated in the stratum radiatum (s.r.). Granule cells (blue) in the dentate gyrus (DG) receive major input from the entorhinal cortex. Granule cells then project to dendrites of CA3-area pyramidal cells (orange) via mossy fibres (MF). CA3 pyramidal cells project to CA1-region pyramidal cells (red) via Schaffer collaterals (SC).

1.2 Glutamatergic Synaptic Transmission

Rapid communication between neurons is achieved through the conversions of electrical and chemical signals at specialized meeting points between two neurons called synapses. A single neuron can form thousands of synapses with other neurons [20]. In brief, transmission begins with the initiation of an action potential at the axon hillock of the presynaptic neuron. The action potential then propagates down the axon until it reaches a presynaptic terminal and causes depolarization of the substructure's membrane. This depolarization opens voltage-dependent calcium channels (VDCC), leading to an influx of calcium. The calcium influx triggers vesicles containing chemical neurotransmitters to release their content into the synaptic cleft by exocytosis [20]. The neurotransmitter then diffuses the short distance across the synaptic cleft and binds specific receptors on the postsynaptic neuron located at specialized protrusions of the dendritic tree known as dendritic spines[20]. Neurotransmitter clearance from the synaptic cleft can be achieved by reuptake of the chemical by the presynaptic terminal, by surrounding glial cells[21], or by enzymes present in the cleft which metabolize the transmitter [22].

In the case of excitatory transmission, activation of postsynaptic receptors leads to opening of ion channels which depolarize the local internal dendritic environment resulting in an excitatory postsynaptic potential (EPSP) [23]. This depolarization is passively relayed through the dendritic tree and can reach the neuron's cell body [20]. If the soma's axon hillock develops a threshold level of depolarization by both temporal and spatial summation of multiple EPSPs received, an action potential may be initiated by the postsynaptic neuron and consequently be propagated down its own axon [20].

1.2.1 Glutamate Receptors

The principal neurons of the hippocampus network are pyramidal neurons that release the excitatory amino acid glutamate [24]. Upon neurotransmitter release of glutamate into the synaptic cleft, a postsynaptic pyramidal neuron receives the message through several types of glutamate receptors. The two major excitatory glutamate receptors present at hippocampal pyramidal cell synapses are the ionotropic receptors α-

amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor (AMPAR) and N-methyl-D-aspartic acid receptor (NMDAR) [25]. Other receptors include the ionotropic kainate receptor [26] and the class of G protein-coupled receptors (GPCR) known collectively as metabotropic glutamate receptors (mGluR1-8) [27, 28].

AMPA receptors and NMDARs are similarly distributed throughout the brain [29]. Colocalization of these receptors has been described in several brain regions including the CA1 hippocampal dendritic spines [26]. Postsynaptic spines are protein rich and specialized regions that contain a wide array of cytoskeletal and scaffolding proteins that regulate synapse structure, receptor localization, and bind and orient proteins required for both downstream signaling and upstream regulation of synapse function. Together, this organized and complex collection of proteins and membrane structures make up the "postsynaptic density" (PSD) [3].

1.2.1.1 AMPA Receptors

AMPA receptors are heterotetrameric glutamate-gated ion channels that contribute to the vast majority of depolarization during glutamatergic excitatory synaptic transmission [30]. AMPAR gene family consists of four distinct yet similar genes which each code for an AMPAR subunit type (GluR1-4), though isoforms are possible. Though subunit affects channel kinetics, AMPARs are generally considered fast-acting and short-lasting, with a short refractory period to recover [31, 32].

Perhaps the most important subunit-dependent mediation of AMPAR function is the role of GluR2 in cation-specificity. GluR2 is one of the most abundant subunits and its presence in an AMPAR renders the receptor essentially Ca2+-impermeable [33]. In the hippocampus, AMPARs are expressed not only on pyramidal cells but also on GABAergic interneurons and glial cells. Pyramidal cells express mainly GluR2-containing AMPARs such as GluR1/2 and GluR2/3, whereas GABAergic interneurons and glial cells may contain more Ca2+-permeable receptor types[34, 35].

AMPA receptors are anchored and organized at the synapse by several scaffolding proteins. Many of these proteins contain protein motifs known as PDZ domains that interact with the intracellular C-terminal tails of the receptor subunits [32]. For example, Glutamate receptor interacting protein (GRIP) and AMPAR binding protein (ABP) bind GluR2 and GluR3 [36]. Other examples of this highly complex regulation of AMPAR function includes Protein Interacting with C-kinase (PICK1), which brings GluR2 into proximity with some forms of PKC [37], and A kinase-anchoring protein (AKAP), which binds GluR1 and may be involved with AMPAR interaction with PKA [38]. Also, NMDARs and AMPARs can be brought into close proximity through synapse associated protein of 97 kDa, which has a Src-homology domain that can facilitate receptor phosphorylation by the Src family of tyrosine kinases [39].

1.2.1.2 NMDA Receptors

NMDA receptors are higher in affinity to glutamate than AMPAR but display much slower kinetics, which may assist in temporal summation during periods of high frequency synaptic transmission [23, 39]. NMDARs likely contribute little to basal levels of synaptic transmission at CA1 synapses [30]. Early studies using glutamate and aspartate as agonist described a region of negative slope conductance in the I-V relation at potentials more negative than -30 mV, indicating some form of voltage-dependence on activation [40]. It was discovered that this was due to a physiological Mg2+ block of the channel, which requires prior depolarization from resting membrane potential to be removed [40-42]. This has given NMDAR a proposed role in "coincidence detection" since it requires both ligand binding and prior postsynaptic depolarization in order to functionally open [3].

Similar to AMPARs, NMDARs are permeable to monovalent cations. However, unlike GluR2-containing AMPARs, NMDARs are also permeable to calcium ions which can affect local kinase activity [39]. As will be discussed, NMDAR's dual role as a coincidence detector and initiator of intracellular calcium signaling makes it an important player in activity-dependent cell processes.

NMDA receptor subunit composition includes two NR1 subunits, and two subunits which can be either NR2 (four subtypes, NR2A-D) or NR3 (two subtypes, NR3A-B) [43]. The NR1 gene contains three regions of alternative splicing and yields 8 protein variants [44]. Regardless of splicing, functional NR1 subunits contain a binding site for the co-agonist glycine [43]. In contrast to NR1, NR2 subunits are all expressed by distinct genes and do not contain a glycine-binding site but rather a site for glutamate binding [43, 45]. NR2A and NR2B are the most common NR2 subunits in the hippocampus and NMDARs can contain in conjunction with the two obligatory NR1 subunits either two NR2A (NR2A-R), two NR2B (NR2B-R) or one of each [46].

NR2A and NR2B subunits confer different receptor physiology. NR2B subunits have been described to stay open much longer than NR2A subunits, though the type of NR1 subunit associated with it and intracellular protein interactions can greatly affect function [43]. Furthermore, both subunits possess long intracellular C-terminal tails that express different phosphorylation sites and domains for protein binding [46]. For example, though Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) can phosphorylate both NR2A and NR2B subunits, it does so at different sites: serine1289 for NR2A and serine1303 for NR2B [39, 47].

NMDA receptors interact with numerous PSD proteins and the associations that NMDARs make through the PDZ-domain containing PSD-95 are particularly important in mediating PSD processes [48, 49]. Through direct association of NMDAR and indirect association of AMPAR via the transmembrane protein stargazin, PSD-95 can bring the two receptors into close proximity [32]. PSD-95 can also couple NMDAR upstream of Mitogen-activated protein kinase (MAPK) pathways [50] and downstream of Src tyrosine kinase function [51]. Phosphorylation plays a crucial role in NMDAR function. Both serine/threonine kinases as well as tyrosines kinases have been shown to modulate channel conductance and the receptors coupling to downstream pathways related to glutamate-mediated synaptic processes[46, 48, 52].

1.3 Synaptic Plasticity

Synapses are able to modify the efficiency with which they transmit information from one neuron to another. This property is known as synaptic plasticity and is the basis of information storage in the brain. New information that becomes coded by synaptic connections in the nervous system require not only activity-dependent strengthening of specific synapses and depression of others but also long-term maintenance [53]. Much of this thought is credited to the concepts of Hebbian synapse theory [54], which is popularly stated as "*cells that fire together, wire together*". Though many mechanisms likely exist that satisfy activity-dependent changes in synaptic connections, the processes of NMDAR-dependent long-term potentiation (LTP) and depression (LTD) are perhaps the most understood.

1.3.1 Long-Term Potentiation

LTP has gained recognition as a neural process required for several different learning modalities [11, 4]. It was first elicited in the anaesthetized rabbit by Lømo in Per Andersen's lab in 1966 [55] and was formally reported in 1973 [56]. To define LTP, it is an observed long-lasting increase in a synapse's efficacy upon considerable activation of that synapse. LTP mechanisms differ across and within brain regions and neurotransmitter type. For example, LTP at the glutamatergic synapses of the CA1 hippocampal region is considered to be Hebbian (requires activation of both presynapse and postsynapse) and NMDAR-dependent [1]. In contrast, LTP in the nearby CA3 region between glutamatergic mossy fibres from the dentate gyrus and postsynaptic pyramidal cells can be non-Hebbian (only presynaptic mechanisms required) and NMDAR-independent [57].

Within the hippocampus, LTP has been extensively studied at the CA1 region; specifically between the excitatory glutamatergic Schaffer collaterals coming largely from the CA3 region and the CA1 cell body dendrites. Performing field recordings of the excitatory postsynaptic potentials produced in the CA1 apical dendritic layer in response to stimulation of Schaffer collaterals is a simple and effective way to examine the effect of high frequency stimulation (HFS) on postsynaptic potentials [1]. The parameter

commonly used to compare field excitatory postsynaptic potentials (fEPSP) before and after a conditioning stimulus is the slope of the fEPSP waveform. A maintained increase in the fEPSP slope is characteristic of LTP induction (Figure 1.2).

The cellular mechanisms mediating LTP in the CA1 hippocampus are complex. However, LTP induced by HFS is thought to be mediated largely by glutamate receptors. Since LTP-induction has been observed to be NMDAR-dependent, the Ca2+ influx may be a key event involved in LTP, with Ca2+ acting as a second messenger. In fact, the expression of LTP involves a number of intracellular signaling pathways which are initiated by a Ca2+ influx. These processes ultimately lead to a maintained increase in synaptic strength to proceeding stimulation by either increasing neurotransmitter release from the presynapse, or by increasing the quantity and efficacy of ionotropic glutamate receptors at the postsynapse by the modification and translocation of already-existing proteins [58].

Several proteins have been implied as downstream signals for NMDAR-driven LTP. The most recognized kinase is CAMKII for several reasons [58]. First, the activation of CAMKII requires only calmodulin and the presence of calcium. Calmodulin is already present in the PSD [59] so once NMDAR activation triggers a calcium influx, CAMKII is readily activated. Second, CAMKII is capable of autophosphorylation upon activation and so can remain active even after the transient calcium influx [60]. Third and lastly, CAMKII has in fact been shown to be activated upon HFS stimulation of CA1 afferents in an NMDAR-dependent manner [61]. CAMKII acts on ionotropic glutamate receptors either directly or through a cascade of events[3]. For example, it can phosphorylate residues on AMPAR subunit GluR1 during LTP [3], which may both enhance the receptor's channel conductance [62] and transport it to the synapse [63, 64] (Figure 1.3).

Other threonine/serine kinases such as PKC and PKA are known to play a role in LTP pathways [58]. For example, PKC inhibitors have been reported to block LTP [65] while increasing PKC activity can enhance LTP [66]. However, the NMDAR-mediated calcium influx may not be sufficient for their activation. Conversely, there are proposed mechanisms for glutamate-mediated activation of PKC that involve synergistic actions of

NMDAR and mGluR [67], but its physiological contributions to LTP are still not clear. It is more likely that PKC and PKA pathways converge either upstream or downstream of NMDAR pathways but are not required during the NMDAR-mediated LTP-inducing event.

HFS paradigms for inducing LTP often produce a short period of heightened short-term potentiation (STP) that diminishes within a few minutes [68]. STP is not a very well understood process. However, it is considered to be NMDAR-independent and largely due to the presynaptic calcium accumulation that results from HFS [68, 69]. This rapid and robust increase may alter vesicle dynamics stimulating the production of more readily releasable vesicles from a reserve pool [70]. Furthermore, the repetitive firing of action potentials can lead to increased calcium influx per action potential [71], which may be in part due to voltage-dependent relief of mechanisms that inhibit calcium channels.

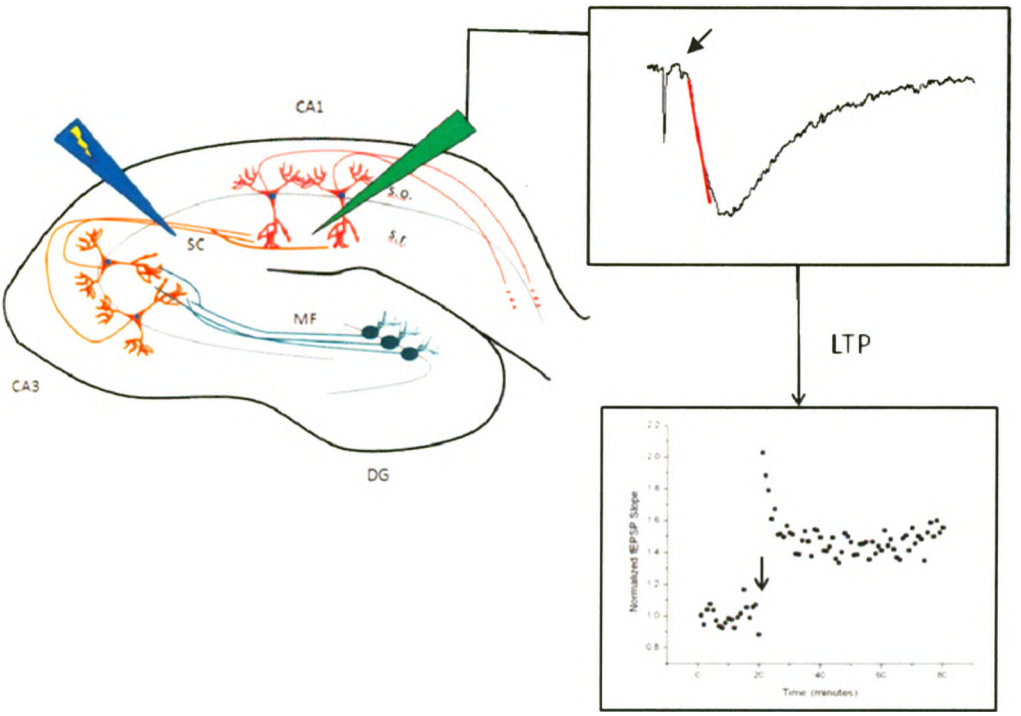


Figure 1.2. LTP Induction. Stimulation of Schaffer collaterals by electrode signals synaptic transmission in the CA1. Extracellular recording electrode placed in the CA1 apical dendrite region records the current sink created by the population of depolarizing dendrites (fEPSP; *top right*). The small waveform immediately preceding the fEPSP represents incoming action potentials from afferent fibres (afferent volley, *black arrow, top right*). HFS enhances fEPSPs. This increase in synaptic efficacy can be quantified by measuring the increase in fEPSP slope (red line) over time (*bottom right*; black arrow represents HFS event).

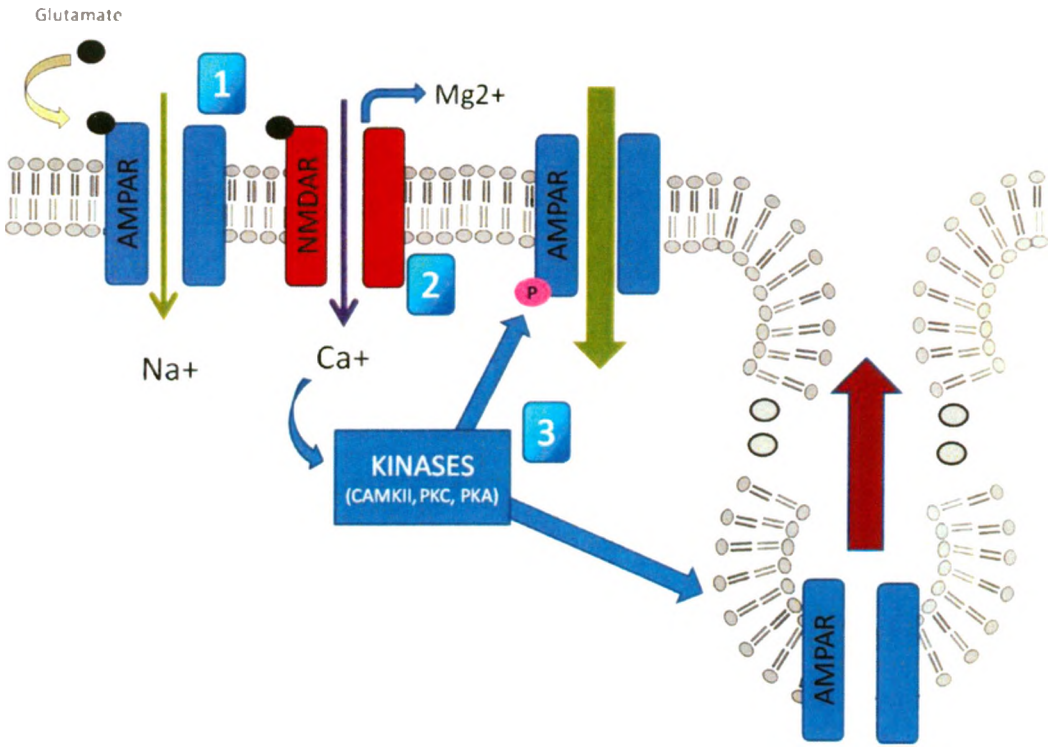


Figure 1.3. Mechanisms of LTP. *1,* Glutamate released by presynaptic terminal binds and opens AMPARs and NMDARs, however NMDARs are still functionally blocked by Mg2+. *2,* Once there is adequate depolarization by AMPARs, the Mg2+ ion is released and NMDARs generate sodium/calcium influxes. *3,* Calcium influx activates of kinases which can then enhance subsequent depolarization by synaptic transmission through mechanisms that increase channel conductance of AMPARs or insert more AMPARs to synapse surface.

1.3.2 Long-Term Depression

If there were only mechanisms for an increase in synaptic efficacy, then synapses would eventually develop a persistently saturated state of potentiation and the ability to store information through pathway-specific synapses would become problematic [3]. LTP's functional counterpart, LTD, is the decrease in synaptic efficacy. The most common induction paradigm for LTD at the CA1 hippocampus involves low frequency stimulation (LFS) of the Schaffer collaterals [52, 72]. Surprisingly, LTD induction is similar to LTP in that they are both NMDAR-dependent [72].

LTD mechanisms are not as well known as in LTP. However, there is a consensus that it involves phosphatase activity that either reverses the effects of LTP-inducing kinases (in the case of "depotentiation") or dephosphorylates other relevant sites on receptors or PSD-proteins related to receptor surface expression [3]. Depotentiation due to the dephosphorylation of residues previously phosphorylated by activity-dependent LTP involves different mechanisms than proper LTD. For example, although S845A knockin mutation of the GluR1 subunit in mice permits depotentiation following LTP induction, LTD is not stably expressed[73].

Two phosphatases that have been discovered to play a role in LTD processes are PP1 and calcineurin, which are involved in GluR1 dephosphorylation [3, 38]. Furthermore, AMPAR internalization is understood to be a major contributor to LTD [38]. Though a clear mechanism has not been discovered, NMDAR activation by a high dose of NMDA can lead to decreased AMPAR surface expression by both inhibiting PKA activity on AMPAR and favouring calcineurin actions [74]. Furthermore, some studies have found that some form of NMDAR-dependent activation of phospholipase C (PLC) leads to a decrease in PSD-95 as well as AMPAR levels in dendritic spines, possibly by Phosphatidylinositol 4,5-bisphosphate (PIP2)-hydrolysis that destabilizes scaffolding proteins that are linked to the membrane by PIP2 [38, 75].

Apart from NMDAR-dependent LTD, LTD can be induced at some hippocampal glutamatergic synapses through activation of mGluR [28]. mGluR-LTD has been identified in various brain regions, and its mechanisms can vary from presynaptic origin to postsynaptic, and Ca2+-dependent to Ca2+-independent (see (28) [28] for review). This form of LTD is not as well understood as NMDAR-dependent plasticity, but is a valuable example of not only the complexity and diversity of synaptic plasticity processes, but also of how GPCRs can affect synaptic physiology.

The overall mechanism controlling LTP and LTD is indeed an immense and complex clockwork. It is clear that NMDAR activation is not a simple "ON/OFF switch" for LTD and LTP. However, since HFS leads to LTP and LFS to LTD, the switch might be dependent on the input frequency and the way the synapse is "tuned up" to receive that input message.

1.3.3 BCM Model of Bidirectional Synaptic Plasticity

In 1982, Bienenstock, Cooper, and Munro (BCM) formulated a computational model for bidirectional synaptic plasticity [76]. The BCM theory proposes that a neuron's synaptic weight changes based on the extent of afferent input (Figure 1.4). If afferent stimulation is greater than a certain value, **ΘM**, then synaptic potentiation will occur; and if it is below **ΘM**, then depression occurs. Thus, **ΘM** represents the LTD/LTP threshold frequency. In fact, Dudek and Bear demonstrated this property in the hippocampus by stimulating slices at different frequencies and plotting the direction and extent of plasticity against stimulation frequency [72]. Furthermore, the theory explains that **ΘM** is due to the biophysical properties of the synapse and thus extrinsic factors, such as neurotransmitter signals, may cause the value of **ΘM** to "slide". These extrinsic factors may be due to paracrine, endocrine, or other neurotransmitters signals that alter the cell's resting physiology [77].

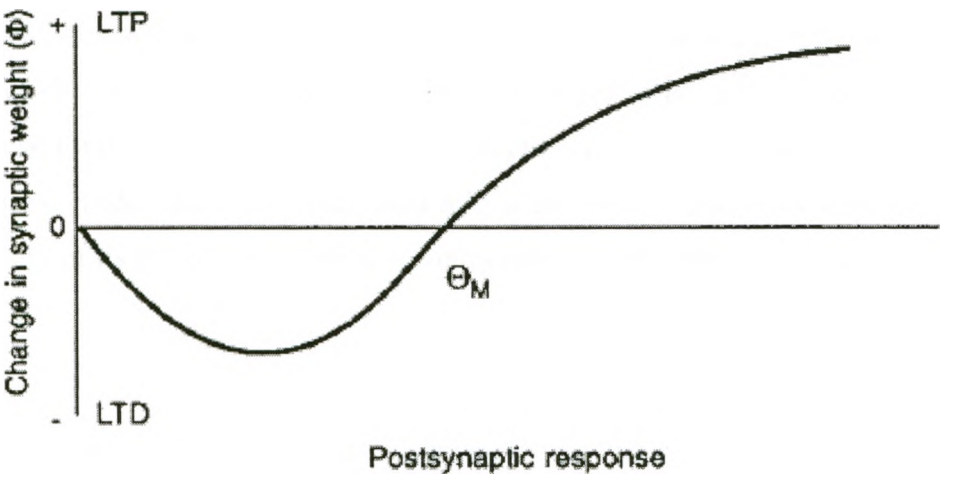


Figure 1.4. Simplified BCM model describing modification of the direction and magnitude of synaptic strength by postsynaptic activity. BCM theory identifies Θ_M as the "switching point": stimulation less than this value produces LTD whereas stimulation greater than this value produces LTP. Figure from Abraham and Tate, 1997[76].

1.4 Cholinergic Expression in the Hippocampus

1.4.1 Basal Forebrain Cholinergic System

The basal forebrain is a structurally heterogeneous brain region located ventral to the striatum and includes the medial septum (MS), the vertical and horizontal diagonal bands of Broca (VDB and HDB, respectively), and the magnocellular basal nucleus (MBN) [8]. Basal forebrain cholinergic neurons project to cortical, limbic and diencephalic regions and are considered to play a particular role in attention, arousal and memory [78], and the diversity of its projections suggest it contributes to many other behavioural states [79].

The secretion and recycling of acetylcholine by cholinergic nerve terminals requires four main protein players: choline acetyltransferase (ChAT), vesicular acetylcholine transporter (VAChT), acetylcholinesterase (AChE), and the high-affinity choline transporter (ChT). Synthesis of ACh in the cytosol involves the addition of an acetyl group to choline by ChAT. ACh is then transported into secretory vesicles by the vesicle-bound VAChT [80]. This process is dependent on a H+/ATPase that generates a proton gradient to power vesicle loading [81]. Upon neurotransmitter release, ACh is broken down into choline and acetate by the extracellular enzyme AChE and choline is taken up for recycling by ChT [82] . The detection of these proteins individually or in combination is a common method of identifying cholinergic terminals [83].

Studies regarding the basal forebrain cholinergic system role in memory have been achieved primarily by pharmacological and lesioning techniques. Early investigations reported that anticholinergic drugs such as scopolamine induce transient amnesia in rats [84]. Similarly, cholinergic stimulation improved the performance of mice

in memory retention exercises [85]. Furthermore, lesioning the basal forebrain in rats resulted in the animals taking longer to master the Morris water maze task [86].

A particularly interesting finding that links cholinergic processes with central nervous system-wide integration for memory function is that ACh levels rise in the cortex and hippocampus following several learning tasks [78, 87]. However, controversy developed in the 1990s when selective cholinergic immunolesioning techniques by injection of the toxin 192-IgG-saporin into the lateral ventricles of rats reported contradicting results. Some findings showed that although there was effective decrease in cholinergic activity in relevant areas such as the hippocampus, immunolesioned animals did not perform differently in spatial learning tasks than spared animals [88]; or that deficits in spatial memory may not be exclusively due to denervation in the hippocampus [89]. However, others reported that spatial working memory deficits were observed in immunolesion models and were linked with hippocampus cholinergic function [90-92].

1.4.2 Cholinergic Expression in the Hippocampus

Cholinergic neurons in the basal forebrain project to the hippocampus mainly from the VDB and MS and innervate the entire dorsoventral aspect of the hippocampus [93, 94]. Both metabotropic muscarinic-type (mAChR) and ionotropic nicotinic-type acetylcholine receptors (nAChR) are present in the hippocampus [95-97]. The muscarinic receptor family is a group of five GPCRs, M1-M5, coded by related but distinct genes. In rodents, all five subtypes have been detected in the hippocampus by various methods including immunocytochemistry [98] and radioligand binding assays [99]. In the CA1 stratum radiatum, M1 receptors are the predominant muscarinic receptor type followed by M4, M3, and M2 [99, 100].

The locus of expression of different muscarinic receptors has been difficult to evaluate in the hippocampus, largely due to the heterogeneity of neuron types present. In addition to the principal glutamatergic pyramidal neurons and inhibitory GABAergic interneurons, extrinsic afferents containing cholinergic, dopaminergic, and adrenergic terminals also exist [101, 102]. The Gq-coupled M1 receptor is well established to be present

at CA1 dendritic spines [19, 98] though has also been shown to be present at the presynaptic terminals of Schaffer collaterals [103] along with M3. Gi-coupled M2 receptors are mainly expressed as presynaptic regulatory autoreceptors at cholinergic terminals. However, they also have heteroreceptor actions on interneurons and possibly on dendrites of pyramidal cells [104-106], though the latter is poorly explored.

Nicotinic receptors are acetylcholine-gated ion channels formed by five subunits. There have been 17 genes identified that encode nAchR. The two main nicotinic receptors expressed in the hippocampus are homomeric α7 receptors and heteromers containing β2 subunits such as α4β2 [107, 108]. Nicotinic receptors have been demonstrated to be present at both presynaptic terminals as well as at postsynaptic apical dendritic spines [96]. These synaptic loci make nAChR a potential glutamate activity-independent source of depolarization from resting membrane potential.

1.4.3 Cholinergic Modulation of Synaptic Processes

Cholinergic pathways are able to modulate synaptic plasticity of glutamatergic principal synapses in the CA1 hippocampus. However, the physiological direction of plasticity and its mechanisms are controversial and unclear. For example, M1 receptors have been shown to colocalize with NMDARs *in vitro* and can potentiate their response to agonists [109]. However, how this contributes to long-term plasticity, especially when one recalls NMDAR function in both synaptic potentiation and depression, is uncertain. Some studies have shown that immunolesioning the basal forebrain in rats does not affect LTP in hippocampal slices [110]. On the other hand, there have been reports that have demonstrated an effect with lesioned adult rats but not infant [111]. *In vitro* pharmacological slice studies have produced insightful, although confounding, findings. Administration of cholinergic agonist in slices has demonstrated that cholinergic activation affects basal transmission in both directions in a dose dependent manner [7, 112]. Nonetheless, even at high concentrations that cause depression of basal transmission there is increased potentiation after high frequency stimulation, indicating cholinergic enhancement of activity-dependent LTP [113]. Another strong case for acetylcholine's

overall enhancing action on hippocampus LTP is the *in vivo* enhancement of LTP in the presence of the AChE inhibitor serine sulfate [6].

1.4.4 Vesicular Acetylcholine Transporter – Knockdown Mice

To investigate the effects of decreased endogenous cholinergic tone, a gene-modified mouse line was recently generated with a knockdown mutation of the gene encoding the vesicular acetylcholine transporter protein (VAChT) [80]. Mice expressing homozygous "knocked down" VAChT gene expression (VAChT-KD) express VAChT at levels of 30% of that of wildtype. Functionally, VAChT-KD mice display impaired ACh release. Electrophysiological measurements of spontaneous vesicle release at the neuromuscular junction reveals decreased amplitude and frequency of miniature end-plate potentials, indicating decreased quantal packaging of ACh into vesicles. In addition, brain microdialysis of cortex and striatum reveals a 30% decrease in extracellular ACh secretion in heterozygous VAChT-KD. Importantly, these mice express deficiencies in learning tasks that require object or social recognition [80]. Furthermore, treatment of heterozygous mice with cholinesterase inhibitors to maintain cholinergic tone in neural tissue reverses the deficits in at least object recognition [114]. Preliminary results (Prado *et al.*, unpublished) indicate that homozygous VAChT-KD mice show no change in protein expression of muscarinic receptors in the hippocampus. However, whether these receptors are sufficiently activated and coupled to synaptic plasticity-related processes is uncertain.

2. Rationale/Hypothesis

2.1 Rationale

The VAChT-KD mice are a valuable tool for studying cholinergic modulation of synaptic plasticity. Since they endogenously express decreased cholinergic tone, they provide a novel approach to understanding the role of acetylcholine in hippocampal synaptic plasticity without the need to perform invasive lesions or for pharmacological administration.

Though the literature continues to be controversial, there are still strong cases that cholinergic effects on the hippocampus support activity-dependent synaptic plasticity, particularly LTP. With decreased cholinergic input, not only may hippocampal LTP be attenuated, but an overall shift in the frequency-dependence of bidirectional synaptic may also be observed.

2.2 Hypothesis

I hypothesize that activity-dependent hippocampal synaptic plasticity in cholinergic-deficient VAChT-KD mice is impaired: that the extent of LTP expression induced by HFS is attenuated and is related to a corresponding shift in the frequency-dependent BCM curve.

3. METHODS

3.1 Slice preparation

All experiments were approved by the Animal Use Subcommittee of the University Council on Animal Care (The University of Western Ontario; see *Appendix*). With the exception of preliminary studies (see Results: *Optimizing protocol for LTD expression: LTD is age-dependent in wildtype slices*) P21-P35 VAChT-KD homozygous mice and their wildtype (C57BL/J6) littermates were used. Mice were anesthetized with isoflurane and decapitated. Brains were isolated and placed in chilled artificial cerebrospinal fluid (ACSF; containing, in mM: 124 NaCl, 5 KCl, 1.3 $MgCl_2$, 2.6 $CaCl_2$, 1.25 NAH_2PO_4, and 26 $NaHCO_3$, and 10 glucose) oxygenated with 95% O_2/ 5% CO_2 carbogen mix. Hippocampal horns were then isolated and mounted in agar along their longitudinal axis and sliced into transverse sections 350μm thick with a Vibratome 1200S (Leica Microsystem, Heppenheim, Germany). Slices were given at least one hour for recover at room temperature in oxygenated ACSF before proceeding to field recordings.

3.2 Electrophysiology

3.2.1 Data acquisition

All recording signals were amplified 1000 times via a MultiClamp 700B amplifier and digitized by Digidata 1440A, acquired at 10kHz and filtered at 2kHz. Data was recorded and analyzed with Clampex 10.2 and Clampfit 10.2 software, respectively. All equipment and software from Molecular Devices Corporation, Union City, USA.

3.2.2 Extracellular field recordings

Hippocampal slices were placed in a recording chamber with continuous perfusion of oxygenated ACSF maintained at 28-31°C at a flow rate of 3mL/min. A concentric bipolar stimulating electrode (Rhodes Medical Instruments, Inc, Summerland, CA, USA; MicroProbes for Life Science, Gaithersburg, MD, USA) was placed on the

surface of the slice over the Schaffer collateral region. Stimulation pulses lasted 0.1 ms for all protocols. A glass recording electrode (1-5Ω) filled with ACSF was placed in the middle of the CA1 region's stratum radiatum. The depth of the recording electrode in the slice was determined by the depth which produced the maximal response. The following tests were then executed:

i) Input/Output

Starting at a stimulating intensity which produced a minimum fEPSP response, extracellular field potentials were recorded at increasing current intensity every 20 seconds until spiking was produced. The slope relating the afferent volley amplitude with the subsequent fEPSP slope produced with increasing stimulation was used to characterize basal synaptic transmission. Slices which spiked at fEPSP amplitudes less than 1.5 mV were excluded from further studies.

ii) Paired-pulse facilitation

To assess facilitation of fEPSP response following an identical stimulus, slices were stimulated using a paired-pulse protocol at a stimulus intensity which produced either 1/2 or 1/3 the maximum, spike-free fEPSP response. Pulses were paired at the following intervals: 10ms, 20ms, 40ms, 80ms, 150ms, 300ms, 500ms, and 1000ms.

iii) Synaptic plasticity: BCM-like curve generation

Baseline measures were established using single-pulse stimulation at 0.05Hz at a current intensity adjusted to produce 1/2 the maximum, spike-free fEPSP response. After establishing stable baseline recordings for 20 minutes, slices were stimulated at either 100Hz for one second to produce LTP, 1Hz for 600s to produce LTD, or at intermediate stimulations of 5Hz, 10Hz, and 20Hz for 600 pulses each. This "plasticity-inducing"

phase was followed by one hour of baseline stimulation. Recordings of the fEPSP slopes from the last five minutes of this hour were normalized to the mean value of the 20 minute "baseline" period of recording and used to determine the extent of long-term plasticity.

For some experiments, the NMDAR antagonist, (2*R*)-amino-5-phosphonopentanoate (AP5; 100μM; *D/L*-racemic mixture; Sigma-Aldrich, St. Louis, MO, USA), was administered for 5 or 10 minutes following baseline recordings and washed out immediately after the plasticity-inducing stimulation protocols.

iv) Synaptic plasticity: cholinergic agonist rescue

Baseline measures were established using single-pulse stimulation at 0.05Hz at a current intensity adjusted to produce 1/3 the maximum, spike-free fEPSP response. After establishing stable baseline recordings for 20 minutes, slices were stimulated at 100Hz for one second followed by one hour of baseline stimulation. Half of the slices used were administered with cholinergic agonist, carbachol (Cch; 200nM; Sigma-Aldrich, St. Louis, MO, USA), for ten minutes following baseline recordings and washed out after tetanizing stimulation.

3.3 Statistical analysis

All statistical analyses were computed using GraphPad Prism 4.0 (GraphPad Software, San Diego, USA) software. Student's t-test was used to compare between groups. Analysis of variance (ANOVA) was used to analyze multiple groups. All data are presented as means ± standard error of mean (SEM). For synaptic plasticity experiments, data are presented as normalized to pretetanus recordings (eg. 1.14 would be interpreted as a 14% increase from baseline). P values were constant at 0.05.

For graphs illustrating synaptic plasticity, data points (slope of fEPSPs, measured between 25% and 65% of the rise) were grouped in one minute bins and normalized to the mean value of the 20 minute "baseline" period of recording.

4. RESULTS

4.1 Synaptic transmission is impaired in VAChT-KD slices

To determine whether there is a difference in CA3-CA1 synaptic transmission between WT and VAChT-KD mice, input-output relationships were assessed by increasing stimulus intensity and comparing the peak of the afferent volley with the fEPSP slope produced (Fig. 4.1A). I/O relationship slopes (units: $(mV/ms)_{fEPSP}/(mV)_{afferent\ volley}$) of VAChT-KD slices (4.94±0.24; n=27) were significantly decreased compared to wildtype (5.92±0.25; n=32 (mice=24) by Student's t-test ($p=0.018$); Figure 4.1B. Data are presented on a scatter plot (Figure 4.1C). This is interpreted as impairment in synaptic transmission since for a given afferent "input" signal, VAChT-KD slices were more likely to produce a smaller fEPSP compared to wildtype.

The observed decrease in synaptic transmission may have either a presynaptic or postsynaptic basis. Paired-pulse facilitation (PPF), an increase in field response to a second pulse generated shortly after an initial pulse, is a presynapse-mediated form of short-term synaptic plasticity [115]. PPF was assessed by varying the interpulse period and measuring facilitation. Figure 4.1D shows a representative recording of PPF at a 40 ms interpulse period.

PPF was similar between the genotypes at all tested interpulse intervals (Figure 4.1E; two-way ANOVA, effect of genotype, $F_{(1,32)}=3.338$, $p=0.068$), demonstrating that impairment in synaptic transmission was not due to modifications of presynaptic vesicle release mechanisms.

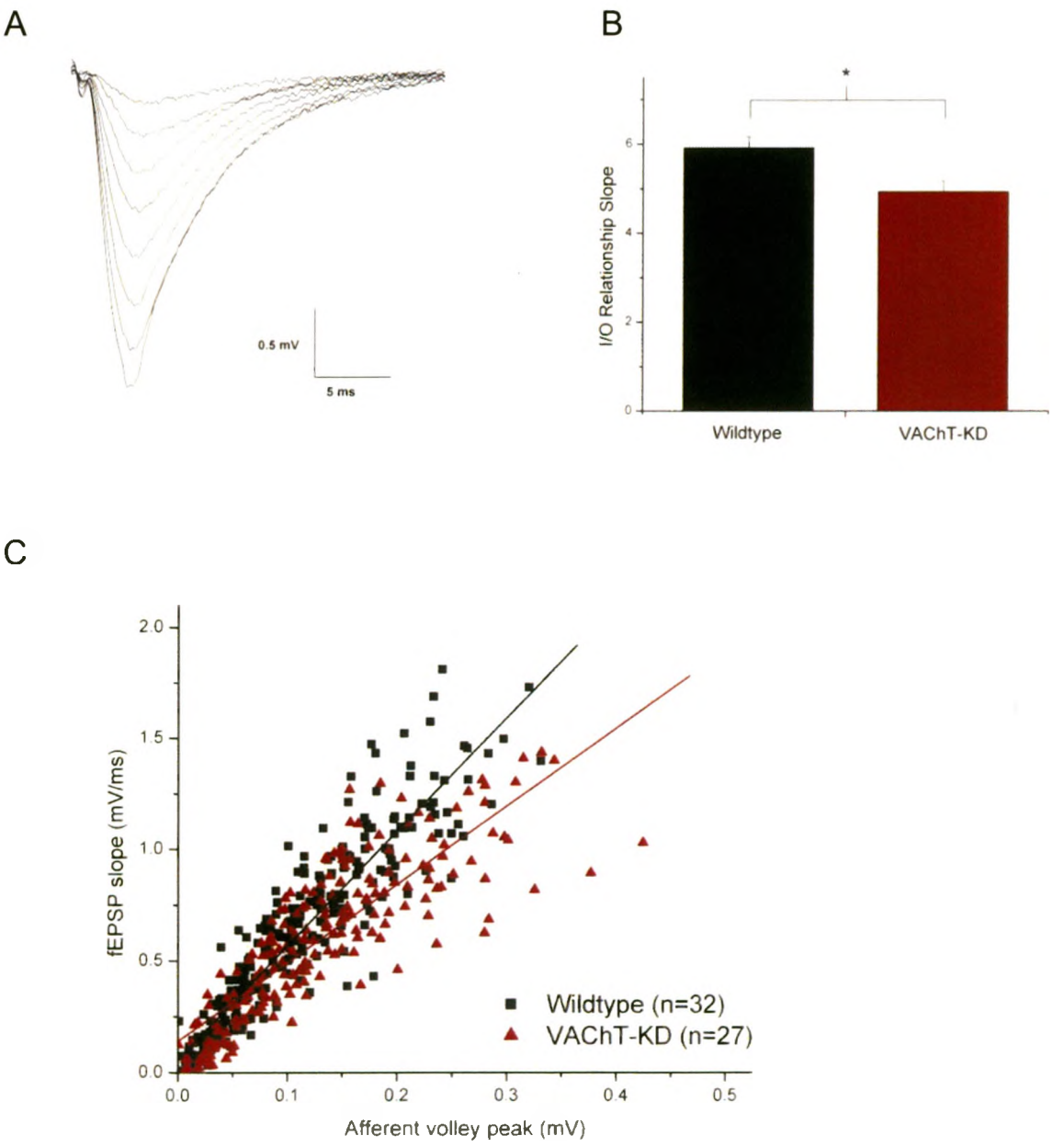
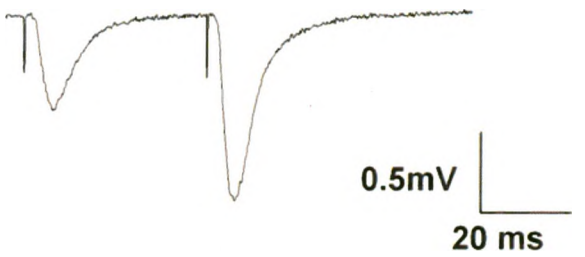


Figure 4.1, A-C. Impaired synaptic transmission in VAChT-KD slices. *A*, Representative traces of input-output relationship produced by increasing the intensity of stimulation. *B*, Bar graph illustrating the decrease in mean I/O relationship slope in VAChT-KD slices compared to wildtype. *C*. Scatter plot of individual I/O points from both genotypes.

D



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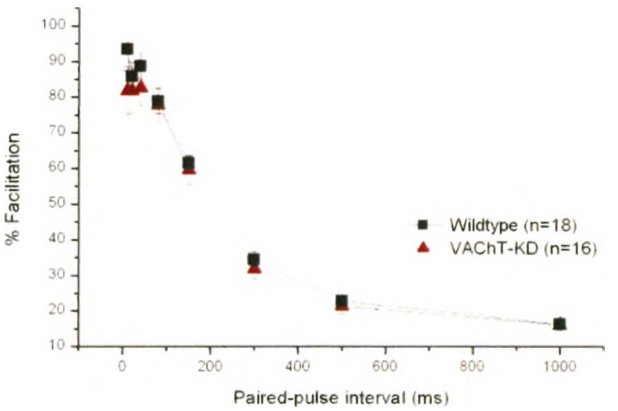


Figure 4.1, D-E. Paired-pulse facilitation. D, Representative trace of PPF with a 40 ms pulse interval protocol. E, Group data illustrating PPF at various pulse intervals in wildtype and VAChT-KD slices.

4.2 Optimizing protocol for LTD expression: LTD is age-dependent in wildtype slices

Initial studies to confirm that activity-dependent LTD can be observed in slice using a conventional 1Hz protocol were performed using wildtype mice that were 35-49 days old. Though short-term depression of synaptic transmission was observed in this age group, slices were not significantly depressed one hour after inducing plasticity (0.978±0.088, n=5 (mice=4); paired t-test against baseline, p=0.075) . Age-dependence of LTD induction by the methods used has been described in the literature [116] and a younger group of mice was studied (P21-P35). Previous LTD studies have used this age group of mice [117]. Preliminary recordings presented significant depression in the younger group (0.751±0.050, n=4 (mice=4); paired t-test against baseline, p=0.015; Figure 4.2).

4.3 Activity-induced bidirectional synaptic plasticity is NMDAR-dependent at CA1 synapses

LTP at CA3-CA1 synapses is an NMDAR-dependent process. To confirm that the synaptic plasticity observed in wildtype control studies is in fact due to NMDAR activation, the NMDAR antagonist AP5 was applied to slices during both LTP-inducing HFS and LTD-inducing LFS.

After establishing stable baseline recordings for 20 minutes, AP5 was administered to the recording chamber for ten minutes. Washout proceeded 100Hz tetanus of Schaffer collaterals. fEPSP slopes of wildtype slices treated with AP5 were not changed during either AP5 application (1.009±0.031, n=6 (mice=6) or the one hour period following tetanus (paired t-tests against baseline, p=0.447 one hour post-tetanus: 0.971±0.035, n=6 (mice=6); Figure 4.3A), indicating that LTP was blocked for one hour after tetanus..

The effect of blocking NMDAR on LTD was confirmed by administering AP5 after 20 minutes of stable baseline recording. After five minutes of AP5 application,

slices were stimulated at 1Hz for 600 seconds followed by immediate washout of drug. Once again, fEPSP slopes were unchanged during drug administration and for the hour following LFS (paired t-tests against baseline, $p=0.156$; one hour post-LFS: 0.955 ± 0.0277, $n=7$ (mice=6); Figure 4.3B).

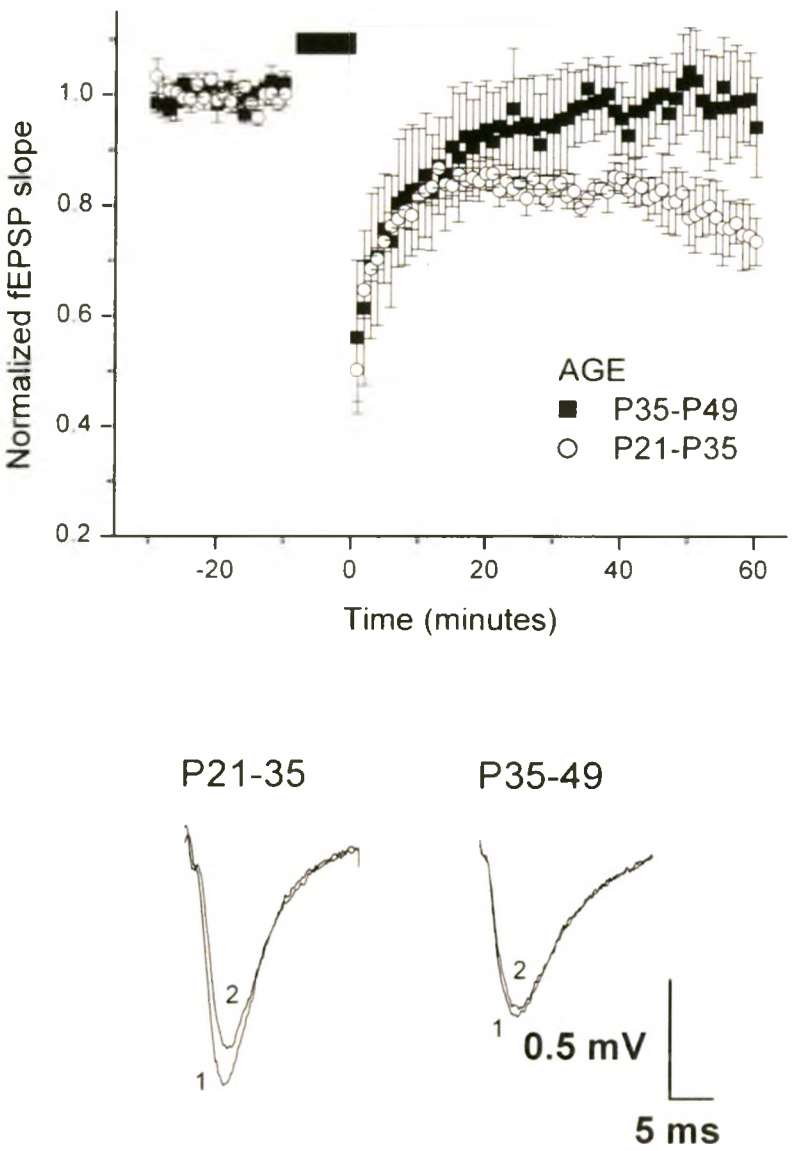


Figure 4.2. Age-dependence of LTD induction. *Top*, Mice age 21-35 days expressed LTD after LFS, while mice age 35-49 days did not. Black bar represents LFS (1Hz, 600 seconds). *Bottom*, Representative traces before (1) and one hour after (2) LFS.

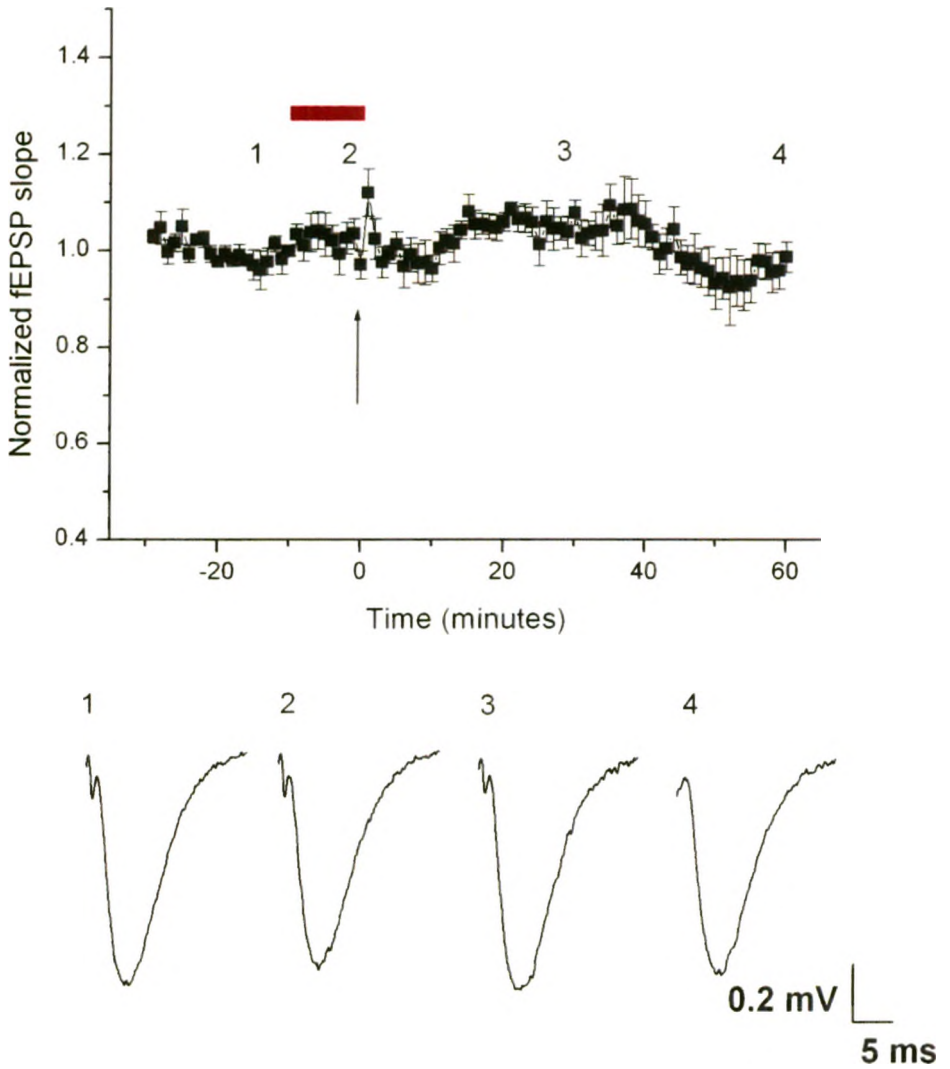


Figure 4.3, A. AP5 blocks LTP. *Top*, NMDAR antagonist, AP5, blocked tetanus-induced LTP for one hour. Red bar represents AP5 application. Arrow represents HFS (100Hz, 1 second). Numbers correspond to representative traces shown *below*. fEPSP slopes are not changed during baseline recording (1), during AP5 application (2), half hour after tetanus (3), and one hour after tetanus (4).

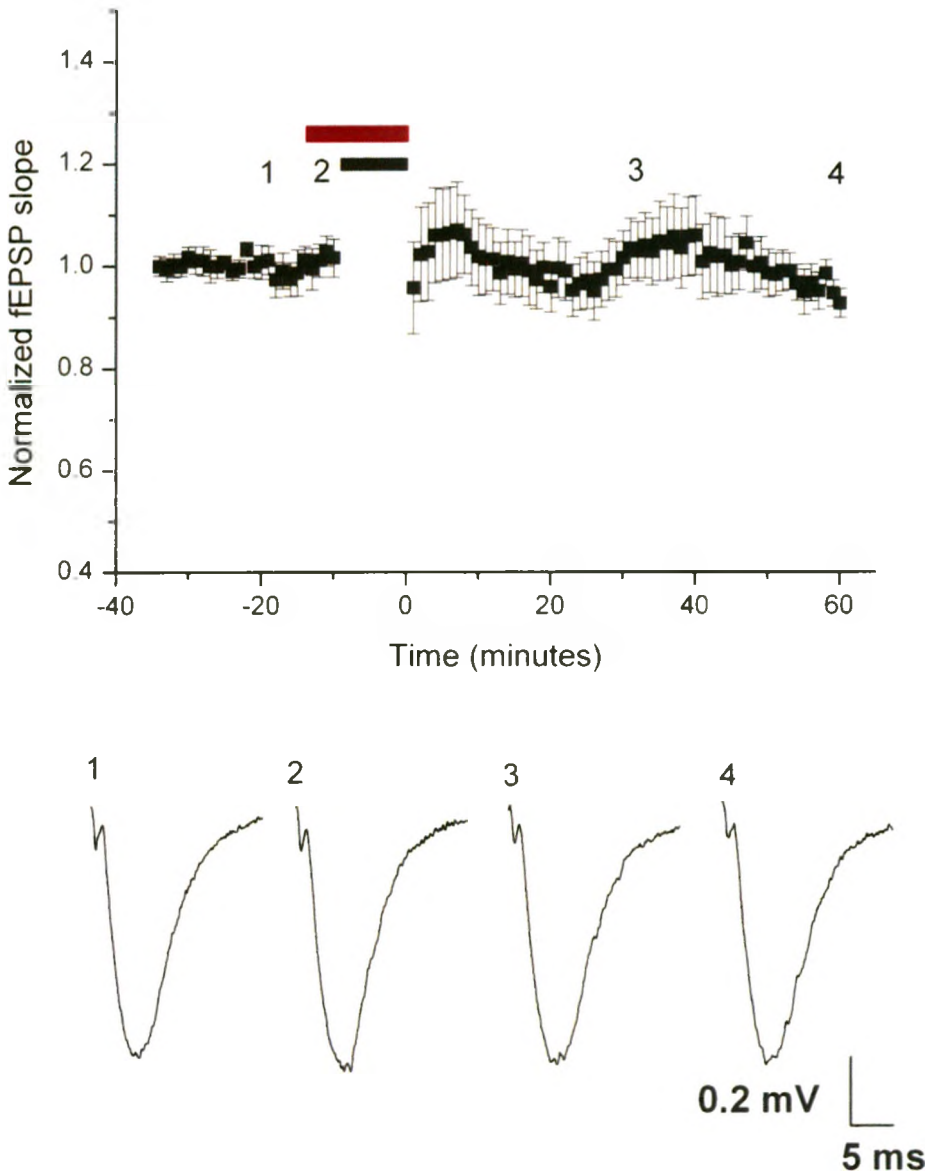


Figure 4.3, B. AP5 blocks LTD. *Top*, NMDAR antagonist, AP5, blocked LFS-induced LTD for one hour. Red bar represents AP5 application. Black bar represents LFS (1Hz, 600 second). Numbers correspond to representative traces shown *below*. fEPSP slopes are not changed during baseline recording (1), during AP5 application (2), half hour after LFS (3), and one hour after LFS(4).

4.4 Hippocampal synaptic plasticity is altered in VAChT-KD mice

4.4.1 LTP is impaired

LTP was induced at the CA1 dendrites by tetanizing the Schaffer collaterals with a 100Hz stimulation protocol for one second (Figure 4.4.1A). Both genotypes produced short term potentiation immediately following tetanus. LTP was quantified by measuring fEPSP slopes collected from the last five minutes of the one hour period following tetanus. Representative traces for each genotype are also presented. LTP was attenuated in VAChT-KD (1.056±0.031, n=5 (4 mice)) slices compared to wildtype (1.347±0.038, n=7 (5 mice); t-test, $p<0.001$). Furthermore, the extent of potentiation observed in VAChT-KD under the experimental conditions was not different to pre-tetanus baseline (paired t-test against baseline, $p=0.14$).

I/O curves and paired-pulse facilitation were assessed before and after LTP induction. The changes in I/O slope observed in both wildtype and VAChT-KD slices reflected the magnitude of potentiation. Following tetanus and one hour of baseline recording, I/O slope increased 34.8±9.8% in wildtype slices (n=4) and only 4.9±3.9% in VAChT-KD slices (n=5) (t-test, $p=0.018$; Figure 4.4.1B). This further demonstrates that the activity-dependent changes that enhance synaptic transmission were attenuated in VAChT-KD slices.

Paired pulse facilitation at various interpulse intervals was also compared before and after tetanus. Though the previously mentioned PPF tests to assess basal presynaptic transmission were performed at an intensity that produced fEPSPs at an amplitude 1/3 the non-spiking maximum, these recordings were taken at 1/2 the maximum amplitude. Since baseline stimulation and tetanus were executed at a stimulus intensity of 1/2 maximum amplitude, to have adjusted stimulation back to 1/3 maximum after potentiation may have been an ambiguous measurement. However, quantitative comparisons of PPF before and after tetanus in wildtype and VAChT-KD slices were not possible at this stimulation intensity because population spikes were produced by the paired pulse (Figure 4.4.1C).

4.4.2 LTD is not changed

LTD was induced at the CA1 dendrites by stimulating the Schaffer collaterals with a 1Hz stimulation protocol for 600 seconds (Figure 4.4.2, top). Both genotypes produce a high magnitude of short term depression immediately following LTD induction and reached a stable state of LTD after one hour. Representative traces for each genotype are also presented (Figure 4.4.2, bottom). LTD was not changed in VAChT-KD slices compared to wildtype (VAChT-KD: 0.773±0.058, n=6 (3 mice); Wildtype: 0.839±0.031, n=7 (5 mice); t-test, p=0.318). It is possible that cholinergic actions on synaptic plasticity are not targeted at LTD-specific pathways.

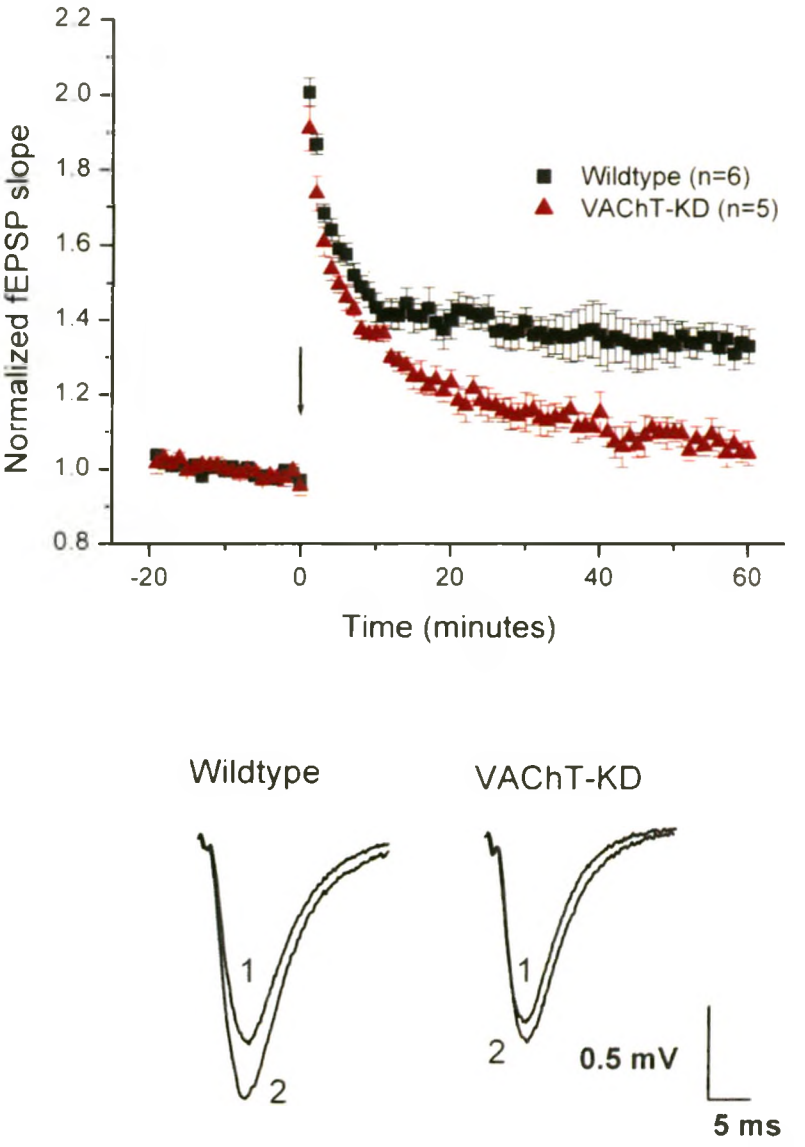
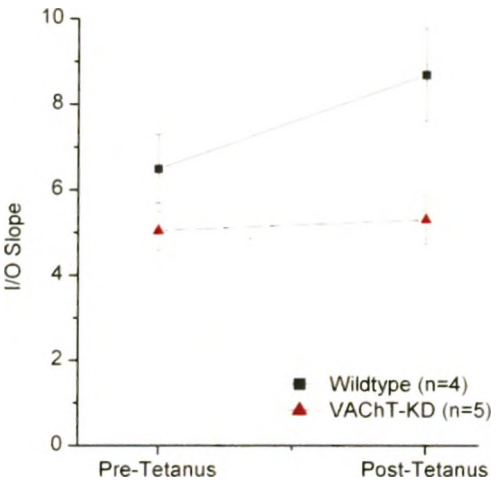


Figure 4.4.1, A. VAChT-KD slices express impaired LTP. *Top*, Group data illustrating changes in fEPSP slope for one hour following HFS in wildtype and VAChT-KD slices. Arrow represents HFS. *Bottom*, Representative traces before (1) and one hour after (2) HFS.



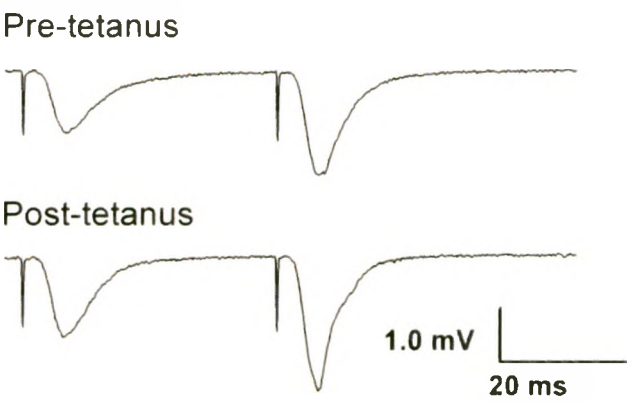


Figure 4.4.1, B-C. *B*, Graph illustrating the change in the slope of I/O relationship before and after LTP induction. *C*, Representative traces of PPF at 40 ms interval in wildtype slices at the intensity that produces 1/2 maximum fEPSP. Spike contamination in after the second post-tetanus pulse made analysis of PPF not possible.

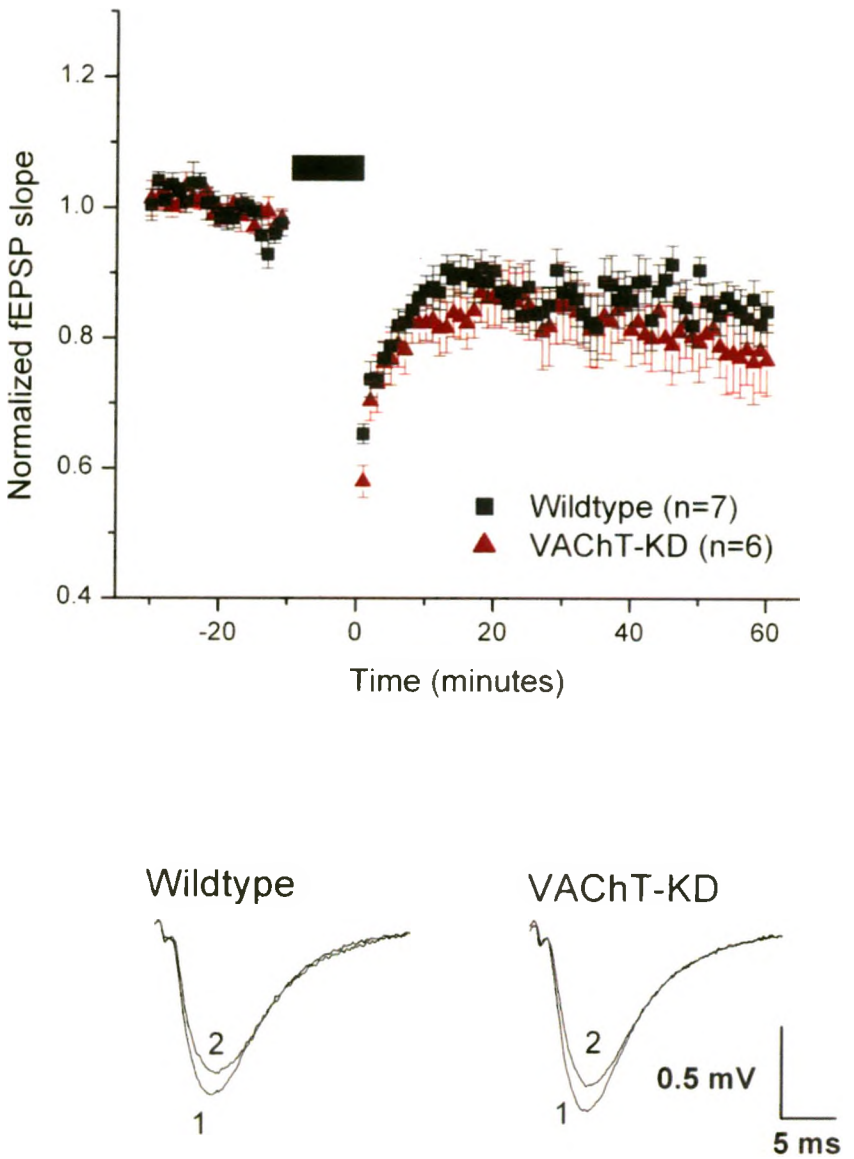


Figure 4.4.2, LTD in VAChT-KD slices is not changed. *Top*, Group data illustrating changes in fEPSP slope for one hour following LFS (black bar) in wildtype and VAChT-KD slices. *Bottom*, Representative traces before (1) and one hour after (2) LFS.

4.4.3 A rightward shift in BCM-like curve

To determine whether there is a change in the frequency-dependent BCM curve in VAChT-KD mice, stimulation protocols at intermediate frequencies (5Hz, 10Hz, and 20Hz) were performed for 600 pulses each. After 5Hz stimulation, both genotypes expressed LTD to similar extents (Figure 4.4.3A; VAChT-KD: 0.820±0.045, n=5 (3 mice); Wildtype: 0.897±0.047, n=5 (5 mice); t-test, p=0.274).

Plasticity-induction using 10Hz stimulation using has a similar effect on both genotypes (Wildtype: 1.144±0.114, n=7 (3 mice); VAChT-KD: 1.021±0.035, n=7 (5 mice); t-test, p=0.319; Figure 4.4.3B). Neither group was potentiated. However, the wildtype slices showed variance that was different compared to VAChT-KD slices (F test to compare variance, $F_{(6,6)} = 10.59$, p=0.011; Figure 4.4.3C). There were several individual wildtype slices that expressed well over 20% potentiation, but also slices that expressed depression.

Synaptic potentiation induced by 20Hz stimulation was similar in slices from both genotypes. Once again, while mean potentiation was greater in wildtype slices, the difference was not different (Wildtype: 1.163±0.058, n=5 (4 mice); VAChT-KD: 1.090±0.084, n=5 (3 mice); t-test, p=0.383; Figure 4.4.3D). Surprisingly, VAChT-KD slices expressed greater short-term potentiation. This was observed in slices prepared from different mice and recorded on different days.

Although the difference in plasticity produced by induction protocols using stimulation frequencies between 1 and 20Hz did not reach significance between wildtype and VAChT-KD slices, the mean potentiation of VAChT-KD slices was consistently lower than wildtype. Plotting mean values of each tested frequency on a logarithmic scale reveals the trend of a downward shift in a BCM-like curve found in VAChT-KD slices (Fig4.4.3E).

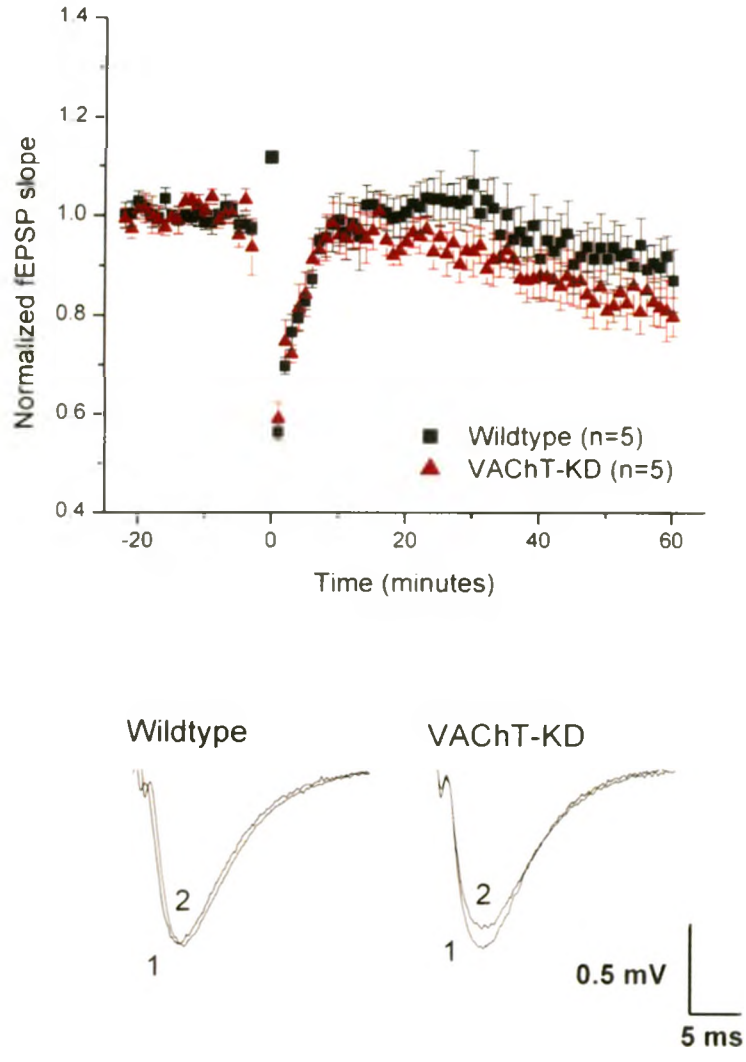


Figure 4.4.3, A. Plasticity induced by 5Hz stimulation is similar in wildtype and VAChT-KD slices. *Top*, Group data illustrating changes in fEPSP slope for one hour following 5Hz stimulation (arrow) in wildtype and VAChT-KD slices. *Bottom*, Representative traces before (1) and one hour after (2) stimulation.

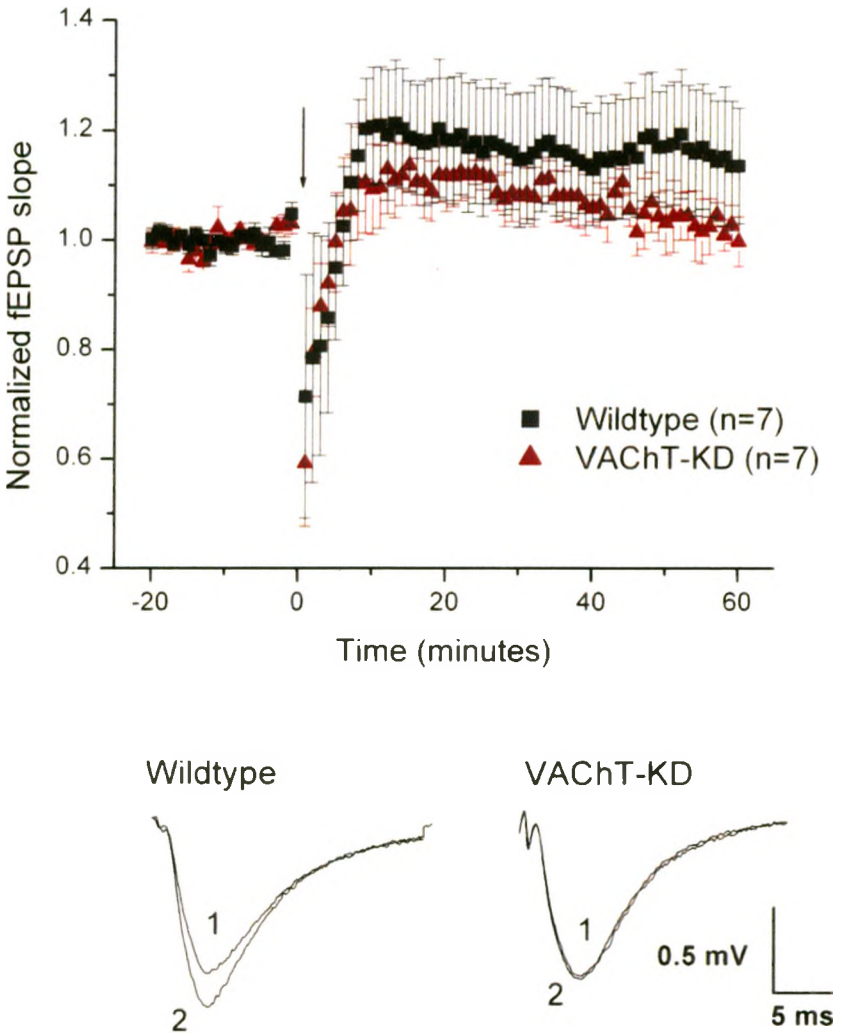


Figure 4.4.3, B. Plasticity induced by 10Hz. *Top*, Group data illustrating changes in fEPSP slope for one hour following 10Hz stimulation (black bar) in wildtype and VAChT-KD slices. *Bottom*, Representative traces before (1) and one hour after (2) stimulation.

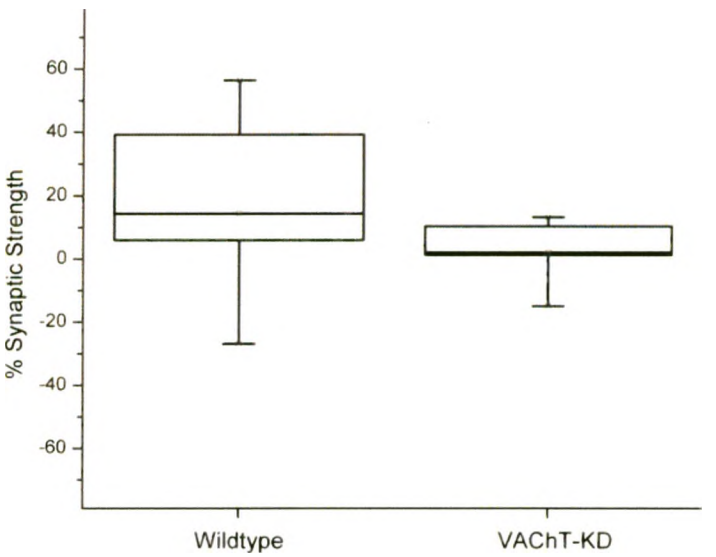


Figure 4.4.3, C. Box-and-whisker plots illustrating difference in distribution of the change in synaptic strength between wildtype and VAChT-KD slices after 10Hz stimulation.

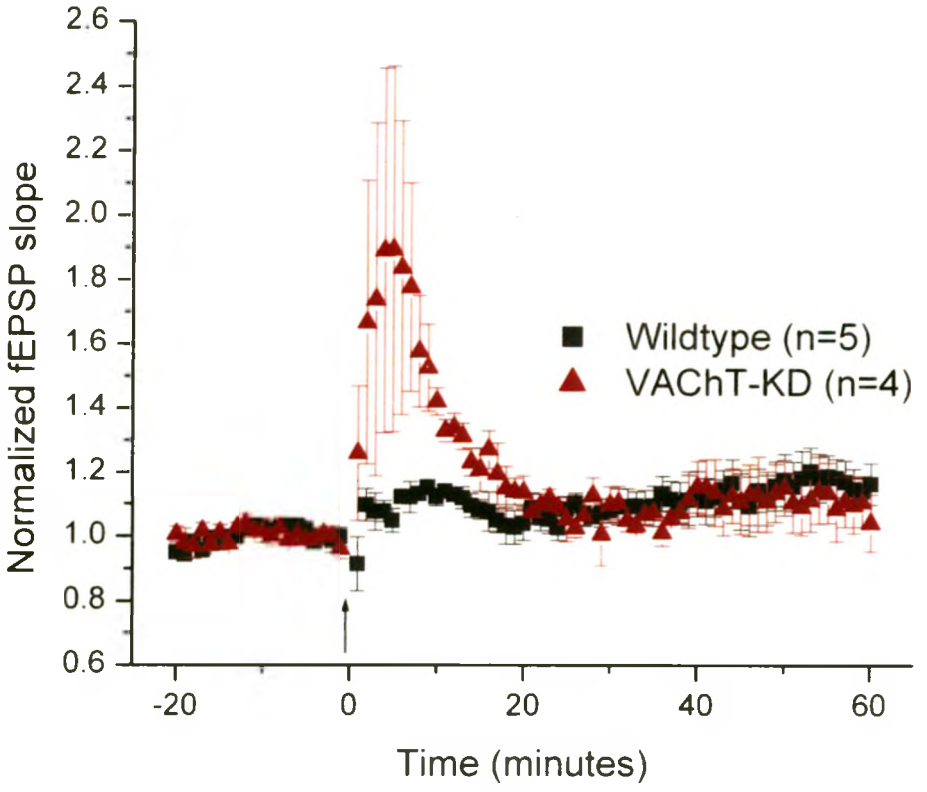


Figure 4.4.3, D. Group data illustrating plasticity induced by 20Hz stimulation (arrow) in wildtype and VAChT-KD slices. VAChT-KD slices expressed enhanced short term potentiation.

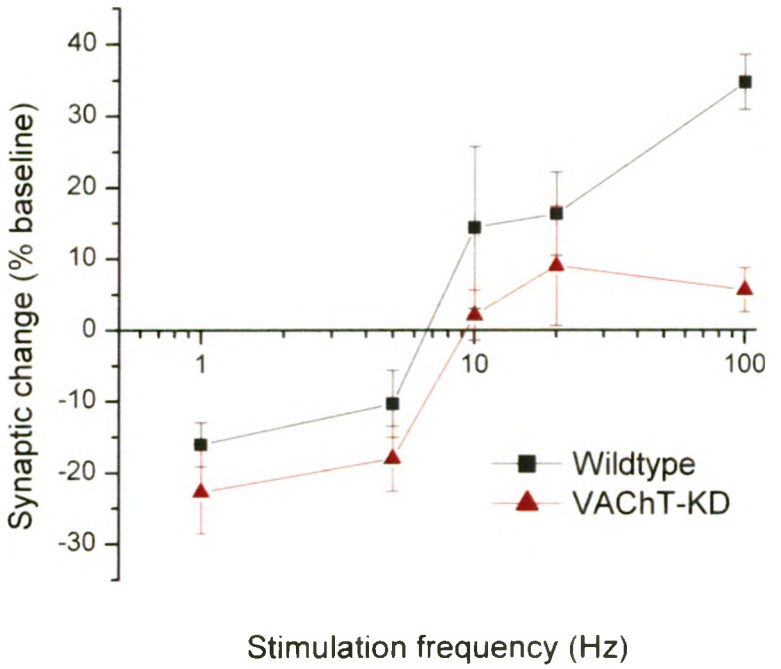


Figure 4.4.3, E. Stimulation frequency-dependence of magnitude and direction of synaptic plasticity in wildtype and VAChT-KD slices.

4.5 Cholinergic agonist rescues LTP deficit in VAChT-KD slices

The possibility that a cholinergic agonist could rescue the LTP-deficit observed in VAChT-KD slices was studied. Carbachol (Cch, 200 nM) was added to ACSF for ten minutes and washed out immediately after 100Hz tetanus. Cch application did not affect basal transmission. In addition to carbachol administration, the experimental conditions differed from the "BCM-curve" experiment in that the stimulus intensity was adjusted to produced fEPSPs 1/3 the maximum amplitude rather than ½. The reason for doing so was to avoid possible spike contamination due to enhanced potentiation.

All slices in this set of experiments produced noticeably greater potentiation using the current procedures compared to the previous set of HFS experiments (compare with Figure 4.4.1A). The reason for this overall increase in synaptic plasticity compared to earlier studies may be due to changes in equipment, stimulus intensity, improved technique, or possibly changes in animal care and handling [118]. LTP-induced recordings of slices from wildtype mice (mice=6) performed at a similar time also expressed increased potentiation and were still more potentiated than VAChT-KD slices (mice=7) (Wildtype: 1.690±0.135, n=7; VAChT-KD: 1.277±0.065, n=5; t-test, p=0.036). Regardless, VAChT-KD still expressed attenuated LTP compared to wildtype controls. Furthermore, administration of Cch enhanced LTP in VAChT-KD slices (VAChT-KD: 1.277±0.065, n=5; VAChT-KD + Cch: 1.630±0.134, n=5; t-test, p=0.046), demonstrating that the LTP deficits in these mice can be rescued by acute stimulation of cholinergic pathways (Figure 4.5A). LTP in wildtype slices were not changed using this concentration of carbachol (Wildtype + Cch: 1.623±0.194, n=6; Figure 4.5B).

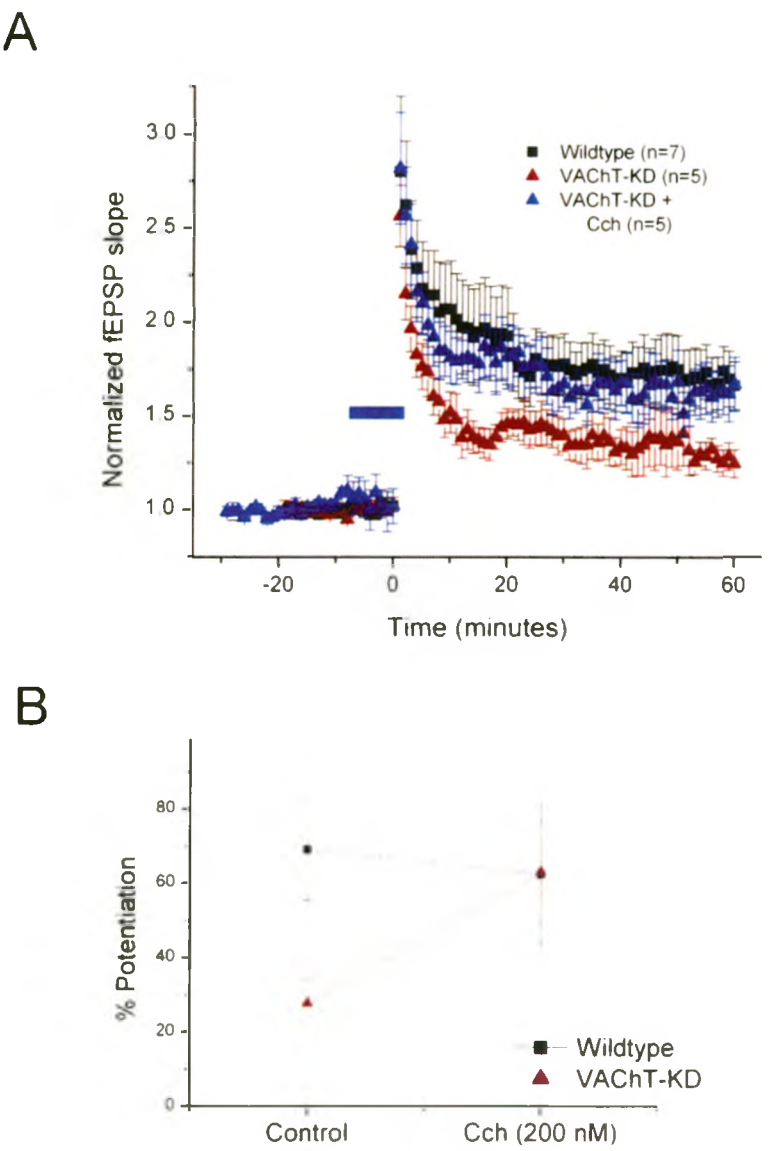


Figure 4.5, A-B. Application of carbachol rescues LTP deficit in VAChT-KD slices. *A*, Group data illustrating plasticity induced by 100Hz stimulation. Blue bar represents Cch application for VAChT-KD slices. *B*, Effect of Cch (200 nM) on facilitation of LTP in wildtype and VAChT-KD slices.

5. DISCUSSION

Overall, the data show both impairment in basal synaptic transmission as well as deficits in synaptic plasticity, as revealed by a downward shift in the bidirectional plasticity threshold and significant LTP attenuation. Unpublished findings by Prado and colleagues show that with the exception of nicotinc α-7 receptor (α7R), glutamatergic and cholinergic receptors are expressed at wildtype levels in the hippocampus of VAChT-KD mice. Although this does not indicate whether surface expression is altered, it does suggest that the machinery required for cholinergic effects on glutamatergic synaptic processes is present, but not activated. The discovery that acute administration of cholinergic agonist rescues LTP in VAChT-KD slices further supports this notion.

5.1 Synaptic Plasticity

5.1.1 Age-dependence of LTD induction

Preliminary studies to optimize LTD induction in wildtype slices led to the observation that hippocampal slices from younger mice more reliably express LTD. Using a wildtype mouse group which included mice between the ages of 5 weeks and 7 weeks old, no change in synaptic plasticity was observed, although short term potentiation was expressed. In contrast, mice between the ages of 3 and 5 weeks expressed LTD and all further studies including BCM-curve generation, carbachol rescue, and assessing basal synaptic transmission used this age group.

Past studies of synaptic plasticity in C57BL/6-background mice have also used the P21-P35 age group [117] and age-dependence of LTD has been previously reported by others [116]. The mechanism for this switch may be related to the developmental switch in PSD proteins that support the expression of NR2A subunits and decrease NR2B expression [119, 120]. At mature synapses, NR2A is generally localized to the synapses whereas NR2B is expressed more extrasynaptically [121, 122]. Furthermore, since these subunits have different C-terminal tails, they bind to PSD proteins differently and can be

modulated differently by upstream kinases as well as can signal different downstream pathways [123, 124].

The effects of NR2 subunits on direction of NMDAR plasticity as well as the overall cell health is a field of growing interest and debate [123]. Controversy regarding this matter may be due to lack of specificity of pharmacological techniques and differences in the brain regions of study. It is likely that either NR2A-R or NR2B-R can signal bidirectional plasticity [125], however, they do so at different efficacies, which may be due to upstream regulation, downstream signaling, or surface localization.

5.1.2 LTP is impaired in VAChT-KD slices

A major finding of this project is that VAChT-KD hippocampal slices express impaired long-term potentiation. It is well understood in the literature that activity-dependent bidirectional plasticity at glutamatergic CA3-CA1 synapses is NMDAR-dependent [58]; and this was confirmed by experiments reported here. Since LTP is NMDAR-dependent, this impairment may be due to downregulated mechanisms upstream NMDAR function. There are several ways by which both muscarinic as well as nicotinic receptors can facilitate activity-dependent LTP by modulation of NMDAR-dependent processes.

M1 muscarinic receptors are Gq-coupled GPCRs and are colocalized with NMDAR in hippocampal pyramidal neurons and can potentiate its actions [109]. Gq proteins are generally upstream of Phospholipase C (PLC) activation and pathways that lead to the upregulation of intracellular calcium. PLC activation leads to the hydrolysis of PIP2 into inositol triphosphate (IP3) and diaglycerol (DAG) [97]. IP3 opens calcium channels expressed on intracellular compartments and causes an increase in intracellular calcium [126]. The combination of calcium and DAG can activate both conventional and novel (calcium-independent but DAG dependent) PKCs at the cell surface [126].

Activation of the serine/threonine kinase PKC is not only a consequence of LTP signaling downstream of NMDAR [58]but it is also an upstream modulator of the NMDAR [46]. Pharmacological application of PKC can enhance NMDA-evoked current in cultured hippocampal neurons [52, 127]. Therefore, since M1 can potentiate NMDAR currents, it may do so through PKC activation. It is possible that in cholinergic deficient VAChT-KD mice less PKC is activated in pyramidal neurons than in wildtype, and molecular assays to detect changes in activated PKC in dendrites of VAChT-KD CA1 stratum radiatum can test this proposal.

Downstream molecules associated with PKC modulation of NMDAR should also be considered to further understand why LTP is impaired in VAChT-KD slices. PKC can regulate NMDAR directly by phosphorylation sites on both NR1 and NR2 subunits [127]. NR2A and NR2B are the only NR2 subunits known to respond to PKC . The literature regarding direct PKC actions remains rather confusing [46]. For example, NR1 phosphorylation of NR1/NR2 heteromers may actually be inhibitory [128]. However, NR2 subunit phosphorylation by PKC is considered to potentiate NMDA-evoked currents either by increasing single-channel kinetics or even inserting more NMDARs at the synapse surface [48]. Investigating the phosphorylation states of these NR1 and NR2 sites in VAChT-KD mice may explain the LTP attenuation observed.

While direct phosphorylation of NR2 subunits by PKC can enhance NMDA-induced currents, indirect enhancements by PKC-mediated kinases have received notable attention as mechanisms for positive plasticity-modulation [46] . The nonreceptor tyrosine kinase family of Src proteins (Src family kinases, SFK) has been shown to be a critical upregulator of NMDAR function [52, 124] through NR2 subunit phosphorylation activity [129, 130]. The prototype member of SFK, Src, is activated by PKC via the intermediate kinase Pyk2 [51, 130] . Activation of Pyk2, along with other anchoring proteins in proximity to NMDARs, stabilizes Src for phosphorylation of NMDAR tyrosine residues. Tyrosine sites on NMDARs for Src phosphorylation have been identified on NR2A [129]. Furthermore, muscarinic stimulation of hippocampal neurons has been shown to potentiate NMDAR channel activity through Src-mediated steps [128].

Though the Src-member of the SFK is activated by PKC pathways, other SFKs may have either distinct or additional mechanisms of activation and action. The SFK Fyn has been shown to phosphorylate important tyrosine sites on NR2B subunits. However, Fyn requires PKA activation to release it from the NR2B-linked scaffolding protein RACK1 [131].

Apart from Fyn activation on NMDARs, PKA plays a considerable role in NMDAR and PSD regulation. PKA phosphorylation on NMDARs can increase its open probability, suppress desensitization to glutamate, and increase calcium permeability [132]. Furthermore, PKA is linked to PSD-95 and placed in close proximity to NMDARs by AKAPs, which are also involved in anchoring the LTD-inducing phosphatases, calcineurin and PP1 [38]. PKA may functionally deactivate these phosphatases either by competing for NMDAR interaction or by directly phosphorylating them [38]. However since LTD was found to be unchanged in VAChT-KD mice, this latter mechanism may not be affected by cholinergic pathways.

Protein Kinase A regulation by muscarinic receptors is generally not well defined in hippocampal pyramidal dendrites, as most of its focus is directed at cholinergic terminals and regions where M2 and M4 receptors, which can downregulate PKA via adenylyl cyclase inhibition [97], are predominantly expressed. M2 receptors are generally considered to not be a major contributor to postsynaptic physiology, and M2 expression on hippocampal pyramidal cells is rarely a point of discussion in the literature. However, the presence of M2 receptors in pyramidal cells [105] and their physiological contributions to potassium channel conductance and LTP in dendrites have been reported [104, 106]. Furthermore, M1-mediated activation of PKA has been described in rat motoneurons [133], but whether this occurs in CA1 dendritic spines is debatable. In support of muscarinic actions that lead to NR2B-targeted tyrosine phosphorylation, it has been reported that carbachol increases NR2B phosphorylation by microinjection into the cortex of rats [134]. Though this finding clearly does not indicate whether PKA is involved, it at least shows that tyrosine kinases interact with NR2B upon cholinergic stimulation. If the PKA mechanisms that regulate NMDAR – whether it is directly or through SFKs – are to be considered as possible pathways of LTP impairment in hippocampal pyramidal neurons

of VAChT-KD mice, the normal muscarinic actions on PKA in this cell-type needs to first be addressed.

Since both LTD and LTP require NMDAR activation in the CA1 hippocampus, the mechanics underlying how NMDAR phosphorylation, or modification of any member of the PSD for that matter, modulates the direction of activity-dependent plasticity is a complex and difficult clockwork to describe. Even if kinase activation by cholinergic agonists occurs and leads to NMDAR potentiation, whether this pathway contributes to LTP or LTD is a current focus on how GPCR mechanisms modulate NMDAR-mediated synaptic plasticity. It has been proposed that NR2A-R-mediated pathways are involved in LTP induction and NR2B-Rs are involved in LTD [120]. Though there exists evidence supporting this divergence of NMDAR mechanisms and its effect on the synapses [123, 135], this model remains controversial [125, 136, 137]. Perhaps the correct model is a synthesis of the opposing theories that considers the ratio of NR2A-R and NR2B-R present at the synapse [125].

In addition to kinase signaling onto NMDARs, M1 receptors also play a role in downregulating the postsynaptic "M current". The M current is a voltage-dependent potassium current generated by the Kv7 potassium channels . Its namesake is from its discovery in sympathetic neurons as a current that can be inhibited by muscarinic receptor activation. In the hippocampus, Kv7 channels have been identified in pyramidal cell dendrites [138]. Inhibiting the M current can increase neuron excitability [139, 140]. At the dendrite, this may translate to facilitated release of the magnesium ion from the NMDAR channel and subsequent functional activation. In fact, pharmacological inhibition of M currents lowers the threshold for LTP induction [140].

The impairment of nicotinic receptors pathways may also play a role in the observed LTP deficit. Blocking α7R with mecamylamine reduces LTP magnitude in mice [141]. Similar to how M1 receptors block the M current, nicotinic receptors may contribute to a synaptic gain in depolarization of the cell membrane potential and thereby facilitate NMDAR opening [108]. α7R expression in the hippocampus of VAChT-KD

mice is decreased (Prado *et al.* unpublished data) and this may play a role in LTP induction. However, the surface expression of α7R is tightly regulated and large internal reserves are maintained at dendritic spines [96]. For this reason, a decrease in protein expression may not translate to altered surface expression. Also, another major finding of this work is that carbachol rescues the LTP deficit observed in VAChT-KD slices to wildtype levels. If surface expression of α7R was decreased, and this was a major source of the LTP deficit, perhaps such a clear and robust rescue would not have been observed.

In addition to molecular mechanisms at the CA1 synapse that may be modulated by ACh, it is important to recognize that the gene-modified mice used in these experiments express decreased ACh secretion throughout their body including the neuromuscular junctions[80]. Furthermore, hippocampal slices prepared from mice placed in an enriched environment show enhanced LTP expression[118]. It is possible that due to motor deficits, VAChT-KD mice were unable to properly explore and manipulate their environment to the same extent as wildtype mice during development, and that this deprivation impaired LTP through pathways more closely related with environmental enrichment.

5.1.3 Metaplastic modulation: Downward shift in BCM curve

The mechanisms proposed above regarding NMDAR potentiation do not affect basal synaptic transmission *per se*. However by modulating NMDAR function, the magnitude and direction of the change in basal transmission that occurs due to NMDAR-activated plasticity can be modified [142]. In the CA1 hippocampus, this plasticity of plasticity, or "metaplasticity", is reported here to be regulated at least in part by cholinergic tone. By stimulating tetanus-naïve slices to plasticity inducing protocols of varying intensity, a trend for a downward shift in the BCM curve was observed in VAChT-KD mice.

Though the 100Hz protocol was the only stimulation intensity to yield a robust change in plasticity, the mean change in plasticity was consistently more negative in

VAChT-KD mice compared to wildtype. The downward shift may be due to the change in balance between LTP- and LTD-promoting mechanisms. It is possible that LTD-inducing pathways are unchanged while LTP-promoting pathways are downregulated. Mechanisms by which LTP may be altered in the hippocampus by cholinergic dysfunction are mentioned above.

After plasticity-induction by the 10Hz stimulation protocol, the variances of the synaptic change were different between the two groups. It is possible that wildtype slices display varying sensitivity to 10Hz stimulation whereas VAChT-KD slices do not due to changes that occur during *in vitro* slice preparation that has a varying effect on endogenous cholinergic tone. Since VAChT-KD mice express endogenously downregulated acetylcholine secretion, hippocampal slices prepared from them are not as sensitive to cholinergic effects. This may also be indicative of the role of cholinergic pathways in modulating the plasticity-threshold point. The slice preparation used in these studies involved isolating the hippocampal horns from the entire brain. Hippocampal slice preparation that preserves innervations from other brain regions such as the medial septum have been described [19]. Though the technique used in the experiments reported here increases the number of transverse slices that could be recording from, perhaps a preparation that better preserves cholinergic innervation would have produced less variable results.

Although VAChT-KD mice express cholinergic dysfunction, there still remains some cholinergic tone. It is possible that this decrease in cholinergic tone is negatively affecting the expression of LTP but is sufficient to maintain normal LTD and intermediate magnitudes of plasticity. Also, in addition to cholinergic receptors, there are several other extrinsic afferents that converge onto similar pathways as muscarinic receptors. For example, the pituitary adenylate cyclase activating peptide (PACAP) receptor, PAC1R is also linked to Gq proteins and can influence synaptic plasticity by NMDAR regulation [130]. Current unpublished findings by MacDonald and colleagues show a BCM curve generated in a similar way to the one reported here but using PAC1R agonist. The results showed that although PACAP agonist does not affect LTP after

100Hz stimulus, there is increased LTP after stimulation at intermediate frequencies. It is possible that this, and additional inputs, are able to compensate for the downregulated cholinergic pathways.

5.1.4 Acute carbachol application rescues LTP deficit

After an LTP deficit and a rightward shift in BCM curve were established in VAChT-KD hippocampal slices, further investigation led to the observation that the cholinergic agonist carbachol rescues this LTP deficit. Carbachol administration has a well-documented dose-dependent effect on basal transmission [7]. Since it was specifically the effects of carbachol on activity-dependent LTP that was of interest, an intermediate concentration of CCh was used that did not alter basal transmission. Cch was applied to both wildtype and VAChT-KD slices for control comparisons. It has previously been demonstrated that even with Cch concentrations that depress basal transmission, tetanus-induced LTP is facilitated by Cch[7]. It was expected that Cch would enhance LTP in both groups, though more so in VAChT-KD slices.

Surprisingly, 200 nM Cch did not affect wildtype slices at all. It is possible that tetanus-induced LTP in wildtype slices is not sensitive to this concentration of Cch. An enhancement of LTP, however, was observed in VAChT-KD slices when tetanus was coupled with carbachol administration. This finding demonstrates that despite chronic and developmental deficits in cholinergic tone, these mice still maintain the machinery required for normal synaptic plasticity. Since LTP is rescued in Cch-induced VAChT-KD and LTP expression ultimately relies on the upregulation of AMPAR physiology at the synapse, a reasonable next step would be to test whether there is an increase in activity-induced AMPAR expression and conductance between Cch- treated VAChT-KD slices and untreated VAChT-KD slices. If there is an enhancement, this may be due to increased post-tetanus activity of CAMKII activity and other signals downstream of NMDARs, or due to increased modulator activity of the kinase pathways upstream of NMDARs.

Since M1 receptors are the most abundant muscarinic receptors at CA1 stratum radiatum dentrites, their stimulation is likely involved in the mechanisms that mediate this enhancement by Cch. In the past, there were criticisms regarding the role of M1 activation in regulating synaptic processes. This was largely due to the belief that cholinergic innervation to the hippocampus terminated in the stratum oriens and that acetylcholine could not possibly diffuse to the stratum radiatum [106]. However, recent evidence not only demonstrates the presence of cholinergic terminals in the stratum radiatum, but that the actions of acetylcholine on M1 receptors expressed by pyramidal neurons may be due to non-specific volume transmission [83]. Furthermore, Sheffler et al. have shown that Cch-induced NMDAR potentiation is blocked by the M1-specific antagonist VU0255035 [143].

This finding correlates well with previous reports that VAChT-KD heterozygotes perform better on object recognition memory tasks when administered cholinesterase inhibitors [114], although it is not proven that this is due to restoring the cholinergic tone to the hippocampus in enhance synaptic plasticity. However, CA3-CA1 synapses have been shown to be potentiated in mice during object recognition tests [144]. Furthermore, activation of M1 receptors by injection of agonists into the brain reverses the spatial memory deficits caused by cholinergic immunolesion [91]. Another related interesting finding is that Doralp and Leung have performed *in vivo* experiments characterizing the effects of the cholinesterase inhibitor eserine sulfate and the M1 blocker pirenzepine on awake and mobile rats [6]. They showed that the eserine sulfate facilitates stimulus-evoked LTP in basal dendrites when the animals were either moving or stationary, and that M1 antagonist blocks LTP specifically when the animals were moving. The finding that M1 antagonist only blocks LTP when the animal is moving may relate to the general cognitive function of the basal forebrain in attention. It is possible that these cholinergic afferents are the neural correlate of an "attention"-related cognitive modality [145].

5.2 Synaptic Transmission

Synaptic transmission was assessed by analyzing the input-output relationships in VAChT-KD and wildtype slices. Both genotypes would start eliciting a "population spike" response at similar fEPSP amplitudes. The complex population spike waveform that is generated at apical dendrites is a well documented observation . Strong enough stimulation at the synapses can generate action potential firing from the cell bodies in the stratum pyramidale. This population action potential firing can be detected by electrodes placed in the stratum radiatum (dendritic layer) and is recorded as a positive-going inflection superimposed over the locally-initiated negative-going fEPSP [146]. Furthermore, spiking at similar fEPSP amplitudes suggests that the slices have similar cell body densities.

The slopes of the input-output curves were significantly decreased in KD slices, indicating that the postsynaptic field response is smaller for a given afferent input. Input-output points relate the sum of action potentials being fired by a population of Schaffer afferents with its consequent postsynaptic response. The observed impairment in synaptic transmission may be due to alterations in any process along the afferent firing-to-postsynaptic depolarization physiological pathway, including presynaptic and postsynaptic changes, or, simply a decrease in synapse density in the CA1 region of VAChT-KD slices.

In theory, I/O relation slope should correlate with synapse density – with more sites for synaptic transmission, action potentials would affect a greater number of terminals , elicit a greater postsynaptic response, and thus generate a larger current sink. However, past studies by Woolley et al. demonstrated no correlation between synapse density and slope of I/O curve [147]. On the other hand, immunolesioning basal forebrain cholinergic neurons in early postnatal development has been shown to decrease CA1 dendritic branching and spine density [148]. To confirm whether dendritic branching is stunted in the CA1 of these VAChT-KD mice, a future study to quantify dendritic architecture by Golgi stain analysis can be performed.

Changes in the baseline efficacy of synaptic transmission in the acute presence of cholinergic agonist are well reported [7], suggesting that although chronic cholinergic deficit may affect dendritic branching, there may be faster-acting mechanisms at play to explain the observed impairment of synaptic transmission. Cholinergic projections to the CA1 hippocampus can affect both presynaptic and postsynaptic structures of the glutamatergic synapse [94]. Studies with the cholinergic agonist CCh describe a dose-dependent effect of Cch on the direction of the change in synaptic transmission in hippocampal slices: high concentration (>1 μM) yields depression that has been proposed to be caused by muscarinic presynaptic mechanisms, whereas low doses (< 0.5 μM) yields potentiation [7]. Though some scientific opinions dismiss Cch-induced depression as not physiological due to the high concentration required to produce it [149], there is increasing literature supporting its physiological role in synaptic maintenance [112].

Both nicotinic [96] and muscarinic receptors [94] are present on presynaptic terminals and may influence neurotransmitter release. Nicotinic receptors, such as alpha-7 receptors, are an action potential-independent source of calcium influx into the terminal. Nicotinic signaling has been demonstrated to increase frequency of miniature excitatory postsynaptic currents as well as increase probability of stimulus-evoked vesicle release [107, 150].

In contrast to reports that nicotinic pathways enhance basal glutamatergic transmission through presynaptic actions, there is evidence of pharmacologically-induced [7, 151, 152] as well as endogenous [153] muscarinic actions that inhibit presynaptic transmission [154, 155]. Though a clear mechanism has not yet been established, this presynaptic inhibition may be due to muscaranic inhibition of presynaptic VDCC [154]. In neuronal [103] cell types, the Gq-coupled M1- and M3-subtypes of muscarinic receptors are described to be involved in VDGCC inhibition. Although it seems paradoxical that Gq activation would not only inhibit VDCC but effectively decrease Ca-dependent vesicle release, a possible mechanism may involve Gq-mediated hydrolysis of PIP2, a membrane lipid that stabilizes VDCCs at the membrane [156, 157].

A simple and common method of assessing presynaptic processes on synaptic transmission is by studying paired-pulse facilitation. The main mechanism of PPF

involves calcium remaining in the presynaptic terminal from the priming pulse. Upon subsequent stimulation, the residual calcium facilitates neurotransmitter release resulting in an increase in postsynaptic response [68, 158]. Synapses with an initially high probablility of release thus have lower PPF since the priming stimulus would leave fewer vesicles available for the second pulse [159]. Assessment of PPF in VAChT-KD slices reported here show no difference in PPF compared with wildtype hippocampal slices. Since cholinergic presynaptic actions seem to have opposing effects on vesicle release, it is difficult to ascertain whether VAChT-KD slices have similar presynaptic functioning to wildtype slices or whether they are in fact impaired, but the muscarinic pathways continue to be "balanced" by the nicotinic actions. Considering that VAChT-KD mice still express some albeit limited cholinergic release, it is quite possible that these pathways are sufficiently stimulated for physiological function. Future comparisons of PPF in VAChT-KD slices in the presence of either a nicotinic antagonist such as mecamylamine or a muscarinic antagonist such as atropine will help further determine whether KD slices display similar presynaptic vesicle dynamics as wildtype.

Though PPF is a reliable and common approach to studying presynaptic vesicle release, it is not a useful measure of neurotransmitter loading into vesicles [160]. Studies that measure the amplitude of miniature excitatory postsynaptic currents (mEPSC) in cultured neurons can detect altered vesicular filling [161]. There is currently no report of cholinergic actions on quantal size of glutamatergic vesicles and the neuronal pathways that regulate glutamate uptake into vesicles remain elusive. In kidney and epithelial cells, PKA has a stimulating role on vacuolar ATPases [162], which are also involved in generating the proton gradient required for vesicular glutamate transporter function. However, whether this occurs in neurons and whether it is modified in cholinergic tone-deficient mice is rather speculative.

The observed indication that synaptic transmission is altered in VAChT-KD slices by decreased I/O slope may be due to postsynaptic changes. As mentioned earlier, there is a well-documented dose-dependence of muscarinic agonist action on hippocampal synaptic transmission. The low-dose potentiation, known as muscarinic LTP (mLTP), has

been demonstrated to be due to postsynaptic mechanisms that involve either the M1 muscarinic receptor [149] and/or the M2-type expressed on interneurons [7, 163].

M2 receptors are not as abundantly expressed on pyramidal cells as M1 and their direct role, if any, on principal neuron processes is not clearly understood. However, M2 receptor's contribution to mLTP may be indirectly through its inhibitory expression on interneurons [7, 163]. It is possible that the impairment synaptic transmission observed in VAChT-modified mice is due to decreased M2 receptor-mediated inhibition of interneurons [101]. To test this would be difficult considering that the direction of M2 actions on synaptic transmission is dependent on neuron type, but may be achieved by field recordings studies that use M2-specific agonist with a low, non-saturating concentration of GABA receptor antagonist such as picrotoxin. Alternatively, whole cell intracellular recordings in cultured slices may allow for further specificity since pyramidal neurons and interneurons display visually different morphologies.

AMPAR expression and kinetics play a major role in stimulus-evoked postsynaptic depolarization. M1 muscarinic receptors are expressed on CA1 postsynaptic dendrites and are known to play an active role in the PSD as a Gq activator, and there are many Gq pathways that can upregulate AMPAR. As mentioned before, phospholipase C activation leads to the hydrolysis of PIP2 into IP3 and diaglycerol, which can then lead to an increase in intracellulat calcium as well as PKC activation. Furthermore, calcium can bind calmodulin and activate CAMKII. CAMKII is a kinase protein that is not only activated in pyramidal CA1 neurons during NMDAR-mediated LTP but also by cholinergic stimulation as demonstrated by carbachol administration to rodent slices [164]. PKC and CAMKII [62] can then signal increased AMPAR channel conductance as well as its insertion to the synapse surface. Though the exact mechanism is still unclear, it may involve PKC phosphorylation of GluR2 which rearranges scaffolding proteins to either transport AMPARs to the surface or maintain receptor stability at the surface [37]. Furthermore, AMPAR receptor trafficking to the surface may be stimulated by PKC-mediated Ras/ERK (extracellular-signalling kinases) signaling [165, 166]. There is also evidence that the gamma-beta subunit of Gi proteins can activate ERK signaling in a Ras-independent way, which reintroduces the role of the M2 receptor as part of a possible

synergistic pathway with M1/Ras/ERK [165]. Regardless of pathway, IP3 activation has been demonstrated to enhance basal transmission by increasing AMPAR EPSCs through a calcium-signalling step [167]. If VAChT-KD synaptic transmission is functionally impaired due to these mechanisms, it would show at the synapse surface. To detect possible impairment in AMPAR expression due to decreased M1 receptor tonic activity, immunohistochemistry followed by high-resolution imaging can be performed on dendritic spines to compare density of AMPAR as well as proportion of subunits. Another possible method would be simply by comparing whole cell current amplitudes in the presence of AMPAR agonist. If there is a functional difference in AMPAR expression in VAChT-KD mice, the upstream mechanisms are worth investigating further. Alternatively PKC phosphorylation may also internalize GluR2, thus increasing the proportion of homomeric GluR1 receptors, which have greater single-channels conductances [3, 168].

It should be noted that although PKC and CAMKII are activated and can enhance synaptic transmission by AMPAR membrane insertion, this proposed muscarinic pathway is different from activity-dependent LTP. Activity-dependent LTP at glutamatergic synapses is a process that enhances synaptic efficacy specifically in response to glutamate. The mechanisms of action for activity-dependent LTP in the CA1 hippocampus also require PKC and CAMKII, but the calcium signal in this case enters specifically through NMDAR-mediated synaptic transmission. mLTP and NMDAR-dependent LTP pathways may certainly converge, and considering that M1 receptor is colocalized with NMDAR subunit, mLTP may involve kinase molecules that can also be exploited by NMDAR-dependent LTP. Lastly, AMPAR insertion by muscarinic mechanisms may in fact facilitate NMDAR-dependent LTP by the increased depolarization that would occur during synaptic transmission [23].

5.3 Short Term Potentiation

With the exception of the 20Hz stimulation protocol, post-tetanus plasticity was similar in VAChT-KD and wildtype. At 20 Hz, PTP was greatly enhanced in recordings with VAChT-KD slices. These recordings were taken from different mice on different days, and were performed blind in regards to genotype. As mentioned earlier, STP is mostly a presynaptic effect. For this reason, it may not necessarily relate to long-term synaptic plasticity; however, it may correlate with other interesting neural and behavioural aspects of these mice.

In the current study, VAChT-KD hippocampal slices were also much more sensitive to 200 nM of carbachol than wildtype, at least in regards to LTP enhancement. The cholinergic receptor pathways that are linked with neuron excitability and synaptic processes may be generally more sensitive to cholinergic agonist in these mice as a form of compensation due to the tonic decrease in acetylcholine present. In addition to learning deficits and the current observation of impaired synaptic plasticity, VAChT-KD homozygous mice are also more susceptible to status epilepticus. In an animal model of temporal lobe epilepsy, administration of the muscarinic agonist pilocarpine induces seizures. VAChT-KD homozygous mice express decreased latency to seizures as well as reduced latency to the development of brain damage when injected with pilocarpine [169].

The role of cholinergic dysfunction in epilepsy is a possible and promising future direction for studies with VAChT-KD mice. The observation that STP may be enhanced in certain stimulation conditions further supports this possibility since STP not only occurs in animal models of induced seizure [170, 171], but antiepileptic drugs attenuate STP [171].

5.4 Future directions

Understanding why and how synaptic plasticity and transmission are impaired in VAChT-KD should be an important focus of future studies with these mice. Proposed experiments are mentioned in the respective sections in this report. To summarize, future

studies should investigate the many possible kinase pathways that regulate NMDARs and whether they are altered in cholinergic reduced conditions; should quantify changes in surface expression and physiology of receptors; and should attempt to isolate the contribution of separate cholinergic receptors on specific functions. Furthermore, investigating the effects of hippocampus-specific administration of cholinergic agonist on the performance of learning tasks will further contribute to the behavioural relevance of the carbachol rescue of LTP in these mice. In addition, the mice's candidacy as an animal model for epilepsy is a new avenue of discovery. Studying how cholinergic actions integrate into the control, and in the case of disease, dysregulation, of neuron excitability may provide new perspectives on the nature of epileptiform activity.

5.5 Conclusions

The work of this thesis has demonstrated that hippocampal LTP is impaired and LTD is not changed in mice expressing deficient cholinergic tone. In addition, acute administration of a cholinergic agonist is capable of rescuing this LTP impairment. Together, these findings suggest a role of cholinergic tone in mediating activity-dependent plasticity at glutamatergic synapses. Furthermore, basal synaptic transmission is also impaired in these mice. Overall, this project demonstrates that acetylcholine is a modulator of synaptic processes and suggests mechanisms by which cholinergic pathways may act on synapse physiology.

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7. APPENDIX

Western

08.01.2010
***This is the 2nd Renewal of this protocol**
*A Full Protocol submission will be required in **08.01.2012**

Dear Dr. **Prado**

Your Animal Use Protocol form entitled:

a) Elucidating the function of striatal cholinergic neurons b) Genetic analysis of cholinergic function c) The role of basal forebrain cholinergic neurons in synaptic plasticity and cognition d) Physiological functions of the cellular prion protein e) DECREASED CHOLINERGIC TONE AND HEART DYSFUNCTION

has had its yearly renewal approved by the Animal Use Subcommittee.

This approval is valid from **08.01.2010 to 08.01.2011**

The protocol number for this project remains as **2008-089**

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.
The holder of this *Animal Use Protocol* is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

c.c. M. Pickering, W. Lagerwerf

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Animal Use Subcommittee / University Council on Animal Care
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