Investigating the Effect of Vesicular Acetylcholine Transporter Overexpression on Central and Peripheral Function

by

Paul Michael Nagy

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Laboratory Medicine and Pathobiology
University of Toronto

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2014

Abstract

Cholinergic neurons use the neurotransmitter acetylcholine extensively throughout the central and peripheral nervous systems. During normal and pathological aging (i.e. Alzheimer’s disease) cholinergic neurotransmission decreases, particularly in the basal forebrain which supplies the cortex and hippocampus with cholinergic input. This thesis investigates the effect of vesicular acetylcholine transporter (VAChT) overexpression on cholinergic function. The biochemical characteristics of cholinergic neurons following VAChT overexpression were measured and the behaviour phenotype of these animals was evaluated. Furthermore, the effect of lifelong VAChT overexpression on the age-related impairment of hippocampal structure and function was determined.

No overt differences in the expression levels of presynaptic cholinergic transcripts and proteins were observed following VAChT overexpression in mice. With this absence of presynaptic compensation, acetylcholine release in the hippocampus was found to be significantly elevated. While this increased level of acetylcholine release was not sufficient to significantly modify peripheral function, we observed behavioural phenotypes conducive to central cholinergic action.
VAChT overexpressing mice exhibited decreased locomotion and enhanced response to novelty in the open field. In addition, these mice displayed a release of exploratory inhibition. These data support the ability of VAChT to modulate cholinergic tone and central cholinergic function.

Using a model of normal aging, VAChT overexpression was found to enhance dendritic ramification in newborn neurons of the hippocampus. An improvement of the precision of spatial memory acquisition was also observed in aged mice overexpressing VAChT. These data provide direct immunohistological evidence that VAChT overexpression augments dendritic expansion in the hippocampus and that VAChT contributes to improved precision of hippocampal-dependant spatial memory.

In conclusion, this thesis demonstrates that VAChT overexpression augments central cholinergic function related to locomotion, response to novelty and hippocampal structure and that these features may in part contribute to superior spatial memory during aging.
Acknowledgments

Working in the Aubert lab has truly been a unique experience. During my years as a graduate student, I was fortunate to work with many bright minds and interesting personalities – it definitely made time pass much too quickly. I want to take a moment and thank those who made a special impact on this project, for without you I would not have reached these goals with the same success.

First and foremost, I am very grateful for the guidance and support of my supervisor Dr. Isabelle Aubert. I have seen the lab grow from a very modest few to a neuroscience research powerhouse, collaborating with more research groups than I can keep track. Throughout this growth, you have empowered me to take the lead on many of these projects, and have always been available to discuss and keep me on track. You have influenced my growth as a researcher, a project manager, and a people manager – skills I will continue to use for the rest of my life.

A special thanks to the members of my advisory committee, Dr. JoAnne McLaurin and Dr. Miles Johnston, and Drs. Evelyn Lambe, Kagan Kerman, Mira Puri and Vania Prado for providing expertise, advice and guidance along the way.

This work could not have been completed without the help of Kelly Markham-Coultes, Stephanie Bell, Lillian Weng, Melissa Theodore and Jonathan Oore. You five supported me and my very old mice and were always there for assistance whenever it was needed. We spent hours isolating neurons, performing immunohistochemistry, or just chatting about experiments and life. You are all amazing people and friends.

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Thank you to the students I have had the opportunity to mentor over the past years, it is very exciting to see you all move on to your exciting paths in science. To the future of the Aubert lab,
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<td>AChE</td>
<td>Acetylcholinesterase</td>
</tr>
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<td>AD</td>
<td>Alzheimer’s disease</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>ATPase</td>
<td>Adenosine triphosphatase</td>
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<tr>
<td>B6</td>
<td>C57BL/6J</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
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<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
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<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
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<tr>
<td>BrdU</td>
<td>5-bromo-2-deoxyuridine</td>
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<tr>
<td>CA</td>
<td>Cornu ammonis</td>
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<tr>
<td>cDNA</td>
<td>Complimentary deoxyribonucleic acid</td>
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<tr>
<td>ChAT</td>
<td>Choline acetyltransferase</td>
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<tr>
<td>CHT</td>
<td>Choline transporter</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
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<td>COS-1</td>
<td>Fibroblast-derived cell line</td>
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<td>CP</td>
<td>Crossing point</td>
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<td>DAB</td>
<td>3,3’-diaminobenzidine</td>
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<tr>
<td>DCX</td>
<td>Doublecortin</td>
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<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>DLB</td>
<td>Dark-light box</td>
</tr>
<tr>
<td>DLG3</td>
<td>Disks large homolog 3</td>
</tr>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>E</td>
<td>Epinephrine</td>
</tr>
<tr>
<td>EC</td>
<td>Entorhinal cortex</td>
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<td>EE</td>
<td>Energy expenditure</td>
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<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
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<tr>
<td>EPM</td>
<td>Elevated plus maze</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>GABA</td>
<td>Gamma aminobutyric acid</td>
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<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>gDNA</td>
<td>Genomic deoxyribonucleic acid</td>
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<td>HC-3</td>
<td>Hemicholinium-3</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>Hydroxyethyl piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>IR</td>
<td>Immunoreactivity</td>
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<td>KCl</td>
<td>Potassium chloride</td>
</tr>
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<td>kDA</td>
<td>Kilodalton</td>
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<tr>
<td>KO</td>
<td>Knockout</td>
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<tr>
<td>LTP</td>
<td>Long term potentiation</td>
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<tr>
<td>MEPP</td>
<td>Miniature end plate potential</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>NE</td>
<td>Norepinephrine</td>
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<tr>
<td>MWM</td>
<td>Morris water maze</td>
</tr>
<tr>
<td>nAChR</td>
<td>Nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>O2</td>
<td>Oxygen</td>
</tr>
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<td>OF</td>
<td>Open field</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>poly(A)</td>
<td>Polyadenalation</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>REM</td>
<td>Rapid eye movement</td>
</tr>
<tr>
<td>RER</td>
<td>Respiratory exchange ratio</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RRP</td>
<td>Readily releasable pool</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>SGZ</td>
<td>Subgranular zone</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>Neuroblast-derived cell line</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline with Tween-20</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>VACHT</td>
<td>Vesicular acetylcholine transporter</td>
</tr>
<tr>
<td>VCO₂</td>
<td>Volume of carbon dioxide</td>
</tr>
<tr>
<td>VO₂</td>
<td>Volume of oxygen</td>
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Dissemination of Work Arising from this Thesis

Chapter 2 was published as:


Chapter 3 was published as:


Chapter 4 has been submitted for publication:

Nagy PM, Aubert I. 2014. Hypercholinergic B6eGFPChAT mice exhibit enhanced dendritic complexity of newborn neurons and improved spatial memory during aging.

Co-authored publications arising during the course of graduate studies (Appendix I):


* For this manuscript, I contributed to the collection and preparation of mouse cortical tissue for ex vivo validation of the cholinesterase assay, and co-authorship of the manuscript.
Chapter 1
Introduction

This thesis investigates the effect of vesicular acetylcholine transporter (VACHT) overexpression on cholinergic tone in B6eGFPChAT transgenic mice. While the majority of the work focuses on cholinergic activity within the central nervous system (CNS), specifically the medial septum and hippocampus, the effects of peripheral VACHT overexpression are also explored.

A vast amount of literature has been generated from the long-standing history of cholinergic neurons. Every effort has been made to ensure this introduction is a succinct review of those studies that fall within the scope of the thesis. First, an overview of cholinergic neurons is presented which outlines the cholinergic elements for the synthesis, vesicular packaging and synaptic release of acetylcholine. This overview is important to understand the characterization of B6eGFPChAT mice and potential sites for presynaptic compensatory regulation that are explored in Chapter 2. The populations and general functions of cholinergic neurons are then briefly described, focusing on cholinergic pathways involved in metabolism, peripheral motor function and anxiety-like behaviour as discussed in Chapter 3. Next, an overview of the role of cholinergic neurons in spatial memory and adult neurogenesis is provided, and explored in more detail through the findings in Chapter 4. Finally, the current state of knowledge related to animal models of VACHT overexpression is explored as a preamble to the B6eGFPChAT mouse model used in this study.

1.1 Overview of Cholinergic Neurons

This thesis evaluates the modulation of cholinergic tone on various physiological and functional outcomes. The site of action that has been used to modify cholinergic tone is the cholinergic neuron. As will be revealed, this thesis exploits the overexpression of the vesicular acetylcholine transporter (VACHT) in a commercially available B6eGFPChAT murine model to enhance cholinergic function. However, the existing literature describes many other components of cholinergic neurons that have been used to modify cholinergic activity. Throughout this thesis, comparisons are drawn between the present work and other studies of cholinergic hypo- and hyperfunction. For that reason, this section of the thesis will offer a general introduction to the
neurotransmitter acetylcholine itself, and the biological elements involved in its synthesis, packaging, release and recycling in cholinergic neurons.

1.1.1 Acetylcholine as a neurotransmitter: historical perspectives

The basis of the neuronal network was developed more than one hundred years ago. Using a silver stain developed by Camillo Golgi in 1873 (Golgi, 1873), Santiago Ramón y Cajal reported that the nervous system consisted of a large number of discontinuous nerve cells (Cajal, 1888). The ensuing “neuron theory” proposed that gatherings of independent neurons are interconnected, subsequently confirmed as units we now refer to as synapses (De Robertis and Bennett, 1955).

Following Cajal’s discovery, the electrical or chemical nature of neurotransmission through synapses was unknown. The discovery of the first neurotransmitter started with the discovery of choline in the 1860s by Adolf von Baeyer and led to his subsequent synthesis of acetylcholine in 1867. In 1906, Hunt and de Taveau suggested that acetylcholine could act as a chemical signal between the vagus nerve and the heart (Hunt and de Taveau R.M., 1906), however the natural existence of acetylcholine had not been confirmed. It was not until 1914, when Sir Henry Dale and Arthur Ewins received an extract of ergot fungus, from which they isolated a compound with potent muscarine-like action (Dale, 1914; Ewins, 1914). Through comparison with acetylcholine, first synthesized 50 years earlier, Dale and Ewins demonstrated for the first time, the natural existence of acetylcholine. A seminal discovery was made shortly after demonstrating the chemical nature of vagus nerve stimulation on the heart of a frog (Loewi and Navratil, 1926). In these experiments, the vagus nerve from an excised beating frog heart was place in physiological solution and stimulated, leading to an expected decrease in heart rate. The physiological solution was then perfused on to a second beating frog heart which exhibited reduced heart rate in the absence of vagus stimulation. Loewi concluded that a substance, which he referred to as “vagusstoff” was released from the vagus nerve terminals to act on the heart tissue (Loewi and Navratil, 1926). This substance was later identified to be acetylcholine (Loewi and Navratil, 1926).

The understanding of the subsequent mechanisms for nerve transmission summarizes decades of work. The synthesis, release, degradation of acetylcholine, along with the reuptake of choline for subsequent acetylcholine synthesis involves the concerted effort of presynaptic cholinergic
proteins. The overview of the process is depicted in Fig. 1-1, and the components are described below in detail.

1.1.2 Synthesis of Acetylcholine

Acetylcholine synthesis proceeds occurring to the reaction:

\[ \text{choline} + \text{acetyl coenzyme A} \leftrightarrow \text{acetylcholine} + \text{coenzyme A} \]

A schematic representation of the presynaptic elements for acetylcholine synthesis, packaging, release and recycling is provided in Fig. 1-1 (Amenta and Tayebati, 2008). In brief, the enzyme choline acetyltransferase (ChAT; Section 1.1.2.1) is responsible for catalyzing acetylcholine synthesis (Amenta and Tayebati, 2008). The reaction is reversible by virtue of hydrolytic degradation of acetylcholine by acetylcholinesterase (AChE; Section 1.1.2.2) where liberated choline contributes to an extracellular choline pool that is reprovisioned to the reaction by the choline transporter (CHT; Section 1.1.2.3) (Amenta and Tayebati, 2008). As will be described in this thesis, much attention has been given to these cholinergic elements in the context of improving or limiting cholinergic function. This thesis, however, focuses on increasing the accumulation of acetylcholine within synaptic vesicles by VACHT (Section 1.1.3) to enhance its release from cholinergic terminals. In the following sections, these cholinergic elements are described in detail, with a particular focus on VACHT.

1.1.2.1 Choline acetyltransferase

ChAT is the enzymatic catalyst for acetylcholine synthesis (Fig. 1-1). ChAT was first discovered by Nachmansohn and Machado in 1943. It was further demonstrated by Lipmann and colleagues in 1947 that ChAT catalyzed the specific transacetylation of choline using the substrate acetyl coenzyme A. In situ localization of ChAT activity was first described using lead salts to form a lead mercaptide precipitate with coenzyme A (CoA), a product of the enzymatic reaction (Burt, 1970). These findings were confounded, however, by the inherent lack of specificity of the reaction. The understanding of ChAT localization was dramatically improved when reliable ChAT antibodies were developed for immunocytochemical detection (Wainer et al., 1984). Cholinergic neurons and pathways were identified using these specific ChAT antibodies, coupled to both immunoperoxidase and immunofluorescent detection, in numerous species (see Section 1.2).
Fig. 1-1. Mechanisms for the synthesis, vesicular packaging, release, degradation and recycling of acetylcholine in cholinergic terminals. (A) Choline is transacylated using acetyl-coenzyme A (AcCoA) through the enzymatic activity of choline acetyltransferase (ChAT) generating acetylcholine. (B) Acetylcholine is packaged into synaptic vesicles by the vesicular acetylcholine transporter (VACHT). (C) Following depolarization, synaptic vesicles are shuttled to the membrane where they release acetylcholine to the synaptic cleft. (D) Acetylcholine binds pre- and post-synaptic receptors until being enzymatically degraded by acetylcholinesterase (AChE) to choline and acetate. (F) Extracellular choline is provisioned to the cholinergic neuron by choline transporter (CHT) for subsequent acetylcholine production.
The complete amino acid sequence of ChAT was first determined after the isolation of the complete complimentary deoxyribonucleic acid (cDNA) sequence of porcine ChAT (Berrard et al., 1987). ChAT was determined to be a single subunit globular protein of approximately 68 kDa. This finding allowed for in situ hybridization studies and determination of ChAT messenger ribonucleic acid (mRNA) in neurons of the CNS. From this work, it was determined that ChAT mRNA is localized to neuronal cell bodies where ChAT protein is synthesized in the perikarya of cholinergic neurons and subsequently transported to nerve terminals by axonal transport (Ichikawa et al., 1997).

The localization of ChAT in the peripheral nervous system (PNS) was initially challenged by the lack of satisfactory staining using conventional ChAT antibodies. It was subsequent revealed that a splice variant of ChAT mRNA encoded an isoform that is preferentially expressed in peripheral nervous tissue (Tooyama and Kimura, 2000). The peripheral and common isoforms of ChAT appear to be differentially expressed and follow unique patterns of intracellular localization (Tooyama and Kimura, 2000).

As will be discussed throughout this thesis, particularly in Chapters 1 and 2, the cholinergic neuron exhibits the ability to regulate the release of acetylcholine through transcriptional and post-transcriptional mechanisms. Previous studies have revealed, however, that these changes are not always predictable. For example, the in vivo depletion of ChAT by 50% causes an induction of CHT (see section 1.1.2.3) mRNA, protein and activity (Brandon et al., 2004). The enhanced CHT activity observed in that study increases transport of choline to the neuron, which acts to maintain normal levels of acetylcholine release and prevent behavioural evidence of cholinergic depletion (Brandon et al., 2004). On the other hand, in vitro studies using the SH-SY5Y neuroblastoma cell line revealed that overexpression of ChAT induces the upregulation of CHT (Matsuo et al., 2011). In that study, the effect on the release of acetylcholine was not evaluated. Finally, cardiac-specific overexpression of ChAT elevated intracellular levels of acetylcholine, however under these conditions, levels of CHT or VACHT were unaffected (Kakinuma et al., 2013).
1.1.2.2 Acetylcholinesterase

Unlike other neurotransmitters (i.e. dopamine, noradrenalin, gamma aminobutyric acid) which are removed from the synaptic cleft through reuptake, cholinergic neurotransmission is terminated by acetylcholine hydrolysis through the enzymatic activity of AChE (Fig. 1-1). The idea of esterase activity in serum was first proposed by Sir Henry Dale in 1914 (Dale, 1914). Experiments by Otto Loewi and colleagues confirmed the chemical nature of neurotransmission and validated the existence of AChE (Loewi and Navratil, 1926).

AChE consists of an invariable core of 534 amino acids that deeply houses a catalytic active site (Massoulié, 2002; Soreq and Seidman, 2001; Zimmerman and Soreq, 2006). The entrance of the active site is flanked by five anionic residues, which in itself is surrounded by ten acidic residues. Two plausible mechanisms for acetylcholine entry into the active site have been proposed. The longest standing utilizes substrate binding to the anionic residues that drives an initial conformational change and facilitates acetylcholine passage through the aromatic border of the active site gorge (Szegletes et al., 1999). The active site then binds choline, positioning the ester at the acylation site where acetate is liberated by a hydrolysis step (Massoulié, 2002; Soreq and Seidman, 2001; Zimmerman and Soreq, 2006). The second model predicts the existence of a “back door” adjacent to the active site, which might permit transit of acetylcholine (Sanson et al., 2011; Sussman et al., 1991). It is likely that both mechanisms exist. Through these mechanisms, acetylcholine is processed by AChE at an extraordinarily fast rate of about 1000-10000 substrate molecules per second (Lawyer, 1961).

AChE is widely expressed in tissues that receive cholinergic innervation. However, AChE expression patterns are not always correlated with levels of ChAT. AChE is also found in regions of the brain that receive little, or no cholinergic input, such as the substantia nigra and cerebellum, suggesting that AChE may play non-classical roles outside of cholinergic neurons. AChE has, in turn, been implicated as a putative cell adhesion molecule, a factor in neurite outgrowth, and a catalyst to amyloidogenesis (Silman and Sussman, 2005).

AChE can be regulated through various cellular and physiological stimuli, and appears to be driven by their association with the membrane as tetramers, or as unbound AChE units (Zimmerman and Soreq, 2006). For example, during embryogenesis there is a gradual shift from levels of unbound AChE to tetrameric AChE in a number of different species (Inestrosa et al.,
Pathological loss of cholinergic innervation leads to reduced AChE activity, and increased levels of unbound AChE in the cerebrospinal fluid in various brain nuclei (Darreh-Shori et al., 2004). As such, the inhibition of AChE has been used to elevate levels of acetylcholine and treat some symptoms during Alzheimer’s disease (AD) (Lanctôt et al., 2003). This approach remains the primary route of care in AD and has driven the development of novel approaches (see Appendix I) to screen and identify next-generation inhibitors of AChE (Veloso et al., 2013). In healthy mammals, various stressors have also been shown to induce increases in AChE, an effect that can last for weeks following the original stress event (Kaufer et al., 1998; Meshorer et al., 2005; Nijholt et al., 2004). Similarly, AChE upregulation is produced by corticosterone, organophosphate poisoning and AChE inhibition both clinically (tacrine and rivastigmine) and non-clinically (pyridostigmine and physostigmine) (Darreh-Shori et al., 2004; Meshorer et al., 2005). AChE also appears to be sensitive to substrate inhibition, as the binding of a second acetylcholine molecule to the anionic residues of the active site gorge slows the rate of acetylcholine hydrolysis (Krupka, 1963).

1.1.2.3 High Affinity Choline Transporter

The transport of choline occurs through choline transporters (Blusztajn and Wurtman, 1983; Haga and Noda, 1973; Kuhar et al., 1973; Yamamura and Snyder, 1972). Two types of choline transport systems can be distinguished by their kinetics of transport. The low affinity choline uptake provides choline that is required by cells for metabolic needs. This transporter is a high capacity, low affinity choline carrier (Kd = 50–100 µM) and is independent of extracellular sodium. The low affinity transporter appears to be ubiquitously present in cells and does not correspond to the distribution of acetylcholine. It is therefore predicted to supply choline destined for phosphatidylcholine synthesis.

Choline supply for acetylcholine synthesis in the brain is derived from three principal sources: 1) free choline from blood plasma, 2) from the hydrolysis of acetylcholine in the synaptic cleft and 3) from the breakdown of phosphatidylcholine in plasma membranes (Blusztajn and Wurtman, 1983). Transport of choline by CHT, and subsequently acetylcholine synthesis itself, is inhibited by the drug hemicholinium-3 (Schueler, 1955).

Despite the understanding that choline is transported through hemicholinium-3 sensitive channels, the identity of the choline transporter has only recently been revealed. Sodium-
dependant transporters, predicted by the *C. elegans* genome project, were screened based on their hemicholinium-3 sensitivity (Okuda et al., 2000). One predicted cDNA sequence exhibited the pharmacological characteristics required for the high affinity choline transporter. Orthologs were subsequently characterized in rat (Okuda et al., 2000), human (Okuda and Haga, 2000) and mouse (Apparsundaram et al., 2001) as having greater than 50% identity and 70% similarity to the predicted *C. elegans* cDNA, and designated CHT (Fig. 1-1). CHT expressed in non-cholinergic COS-1 cells allows for choline transport in vitro (Apparsundaram et al., 2001; Okuda et al., 2000). Furthermore, the displacement of radiolabeled hemicholinium-3 from CHT-expressing COS-1 cells by choline provided the first evidence that CHT was not only a high-affinity choline transporter, but also the hemicholinium-3 binding site (Okuda et al., 2000).

CHT−/− mice are not viable and die typically within an hour of birth (Ferguson et al., 2004). Histological analysis revealed depletion in the number of aerated alveoli in the lungs, which suggests that the mice die due to failed cholinergic neurotransmission at the neuromuscular junction at the diaphragm and intercostals muscles that support respiration (Ferguson et al., 2004). On the other hand, CHT+/− mice are indistinguishable from wild type mice. Specifically, the levels of hemicholinium-sensitive choline transport are equivalent despite having 50% less total CHT protein (Ferguson et al., 2004). These findings are explained through the post-translational regulation of functionally active CHT at the synaptic membrane, in that membrane bound CHT levels were equal between CHT+/− and CHT +/+ mice. The mobilization of CHT depends on the cellular requirements for choline transport. In scenarios of high choline demand (i.e. long-term electrophysiology recording or intraperitoneal injection of hemicholinium-3), the hypocholinergic phenotype of CHT+/− is revealed, presumably as CHT stores are depleted and no additional CHT can be mobilized to the synaptic membrane (Ferguson et al., 2004; Lund et al., 2010).

### 1.1.3 Vesicular Packaging and Release of Acetylcholine

#### 1.1.3.1 Quantal Acetylcholine Release

At the beginning of the 1950s, the observation of spontaneous vesicle fusion to synaptic membranes became the fundamental basis for quantal neuroscience. The first work involved studies of postsynaptic electrophysiology of frog muscles, where small monophasic potentials with constant amplitude were observed (Fatt and Katz, 1952). The potentials were similar to the
electrophysiological profiles of end plate potentials that had been described many years before, and were thus referred to as miniature end plate potentials (MEPPs). Using curarine (as an acetylcholine antagonist) and prostigmine (as an acetylcholine agonist), these MEPPs were predicted to arise from localized release of acetylcholine (Fatt and Katz, 1952). At first, it was reasoned that MEPPs were the result of slow, continuous acetylcholine leakage by random collisions of single acetylcholine molecules with the end plates. This hypothesis was later refined to suggest that a MEPP represents the aggregate of thousands of acetylcholine molecules, termed quanta (Fatt and Katz, 1952). Around the same time, electron microscopy was introduced to the study of synaptic ultrastructure, which revealed clusters of small, clear vesicles near presynaptic membrane (De Robertis and Bennett, 1955). This circumstantial evidence suggested that vesicular structures accumulate at specialized sites of the presynaptic terminal and led researchers to believe that acetylcholine may be contained within these vesicles. The proposed ‘vesicle hypothesis’ predicted that acetylcholine is released in an ‘all-or-none’ manner when vesicular and axonal membranes coalesce (Birks et al., 1960). Most evidence concerning evoked acetylcholine release supports the vesicle hypothesis (Heuser et al., 1979) and it is generally accepted by neurobiologists.

1.1.3.2 Storage of Acetylcholine

Pivotal support of the vesicle hypothesis originated from studies using differential and density-gradient centrifugation revealing that acetylcholine in nerve endings were bound within vesicles (De Robertis et al., 1963; Whittaker et al., 1964). It has been estimated that approximately 6000 acetylcholine molecules are contained within a 50 nm wide vesicle (or quantum) (Kuffler and Yoshikami, 1975). When an action potential is propagated to the nerve terminal, the influx of calcium causes the release of multiple quanta producing a depolarization event that resembles a MEPP, but is greater in amplitude. Supra-threshold endplate potentials trigger action potentials in the target tissue.

1.1.3.3 Vesicular Acetylcholine Transporter

Acetylcholine, a quaternary amine, does not readily pass through phospholipid bilayers. Synaptic vesicles therefore require a specific transporter for the uptake of acetylcholine. Evidence for a specific transporter of acetylcholine originated with studies using the pharmacological agent vesamicol. Early results using vesamicol demonstrated its inhibitory effect on rapidly stimulated
nerve-muscle preparations, although the pre- or post-synaptic site of action was not clear (Brittain et al., 1969). Subsequent studies using exogenous cholinergic agonists revealed little or no inhibition by vesamicol, suggesting that vesamicol acts presynaptically (Gandiha and Marshall, 1973; Marshall, 1970b). Consistent with this hypothesis, AH5954, a quaternary analogue of vesamicol, exhibited decrease inhibition due to its inability to permeate the presynaptic terminal (Marshall, 1970a). It was therefore concluded that vesamicol was binding intra-pre-synaptically, acting to block acetylcholine storage (Marshall, 1970a; Prior et al., 1992).

In early work using isolated synaptic vesicles, attempts to demonstrate acetylcholine transport were unsuccessful (Prior et al., 1992). However, a pivotal finding linked acetylcholine transport to adenosine triphosphatase (ATPase) activity (Koenigsberger and Parsons, 1980; Rebois, 1980; Toll and Howard, 1980) and provided the basis for the two-step model of vesicular transport: acetylcholine is concentrated within synaptic vesicles in exchange for protons, which are supplied by vesicular membrane proton pumping ATPases. With this model, vesamicol was later demonstrated to bind to an allosteric binding site within an acetylcholine transporter ‘complex’ (Bahr and Parsons, 1986), later revealed to be the VAChT protein (Alfonso et al., 1993; Roghani et al., 1994).

The precise three-dimensional structure for VAChT has not been resolved, however a three dimensional model has been hypothesize based on structural information of related transporters (Khare et al., 2010; Vardy et al., 2004). VAChT is predicted to contain 12 transmembrane domains that fold into two bundles with N-and C-terminal regions directed towards the cytoplasm. This model forms a central pathway for acetylcholine passage with a rocker motion of the bundles exposing the substrate binding site to the cytoplasm or the interior of the synaptic vesicle (Vardy et al., 2004). VAChT exchanges two luminal protons for each molecule of cytoplasmic acetylcholine, generating synaptic vesicles that contain acetylcholine levels 100-fold greater than the cytoplasm (Parsons, 2000). Given that the available free energy from two protons predicts this gradient to be approximately 3000-fold (Parsons, 2000), acetylcholine storage by VAChT is presumed to be regulated. In addition, the number of acetylcholine molecules per vesicle depends on the number of VAChT molecules per vesicle (Song et al., 1997), suggesting that the level of VAChT may be rate limiting to the release of acetylcholine.

VAChT is predominately localized to synaptic vesicles. Small changes in the expression or localization of VAChT may have the potential to change synaptic transmission, as the amount of
acetylcholine released by a single vesicle does not seem to be sufficient to saturate post-synaptic receptors (Hartzell et al., 1975). In support of this, studies that have used vesamicol and vesamicol analogues to inhibit acetylcholine transport have demonstrated decreased acetylcholine release from synaptic terminals (Searl et al., 1990; Whitton et al., 1986). On the other hand, in vitro studies that have overexpressed VACHT in neurons have demonstrated that increased VACHT expression increases the magnitude and frequency of miniature excitatory currents by increasing the number of vesicles capable of sequestering acetylcholine (Song et al., 1997). The number of copies of VACHT per synaptic vesicle is not known, although studies using the Torpedo predict that rodent synapses contain one to three transporters per synaptic vesicle (Bahr and Parsons, 1986). For this reason, it is reasonable to predict that in conditions of VACHT deficiency, some synaptic vesicles are devoid of acetylcholine. Consistent to this hypothesis, electrophysiological analysis of VACHT-knockout (KO) mice detected small-amplitude, low frequency MEPPs in neuromuscular junction preparations (de Castro et al., 2009a). Furthermore, the release of newly synthesized acetylcholine from brain synaptosomes is completely abolished in VACHT-KO mice (Prado et al., 2006).

The dependence of cholinergic tone and physiological development on VACHT has been demonstrated in mouse models, and is described in detail in Chapters 2 and 3.

1.1.3.4 Heterogeneity of Acetylcholine Storage Pools

It is known that intraneuronal acetylcholine populates distinct storage pools with unique properties for release. The first demonstration of this followed the observation that treating sympathetic ganglia preparations with radiolabeled choline labeled the entire population of ganglionic acetylcholine, and subsequent stimulation of these preparations, in the absence of radiolabeled choline, produced lower than expected extracellular specific radioactivity (Birks and MacIntosh, 1961; Collier, 1969). It was speculated that new acetylcholine was incorporated into synaptic vesicles close to the synaptic membrane, and that these loaded vesicles were the first to be released (Collier, 1969). Subsequent work demonstrated the existence of at least two vesicular fractions in the Torpedo electric organ, named VP1 and VP2 (Prior and Tian, 1995; Zimmermann and Whittaker, 1977). VP1 fractions were found to be slightly smaller and more metabolically active, rapidly accumulating acetylcholine and acting as reserve pool ready to be mobilized for release (Whittaker, 1993). The VP2 fractions are those that support immediate
release and recycling (Whittaker, 1993). Supporting multiple fractions, the application of vesamicol, selectively inhibiting the uptake of acetylcholine to synaptic vesicles, did not affect the rate of acetylcholine release during initial stimulation, but attenuated release during prolonged stimulation (Collier et al., 1986). These findings predict that acetylcholine had been previously packaged into synaptic vesicles poised for release, and that once emptied of their contents, vesamicol prevents vesicle refilling.

Our understanding of synaptic vesicle pools, the accumulation of acetylcholine and the recycling of vesicular fractions has advanced substantially in recent years. Studies in many different species have revealed the existence of three major synaptic vesicle pools, commonly referred to as the readily releasable pool (RRP), the recycling pool and the reserve pool (reviewed in Rizzoli and Betz, 2005). The RRP consists of synaptic vesicles that are docked at specific release sites and are immediately released upon stimulation. The recycling pool consists of synaptic vesicles that recycle upon moderate stimulation. The reserve pool consists of synaptic vesicles relatively reluctant to be released during stimulation (Rizzoli and Betz, 2005). Understanding the dynamics between the three pools has received attention. Studies using supraphysiological stimulation predicted that the reserve pool was recruited once the RRP and the recycling pool were depleted (Harata et al., 2001a; Harata et al., 2001b; Opazo et al., 2010; Schneggenburger et al., 1999). However, physiological frequencies of stimulation have revealed less of a distinction between the recycling and reserve pools (Hayashi et al., 2008; Ikeda and Bekkers, 2009). With these data in mind, Denker and Rizzoli (2010) have proposed that the recycling pool may be re-recycled, before recruitment of reverse vesicles, and that they will eventually mature into reserve vesicles of a timescale of minutes or hours. Reserve vesicles randomly exocytose, and re-enter the cell with a recycling status (Denker and Rizzoli, 2010). As neuronal simulation does not induce vesicle mobility, only their ability to fuse, the RRP pool represents those recycling vesicles within proximity of the synaptic terminal upon stimulation.

Hippocampal synapses contain approximately 200 synaptic vesicles (approximately 5% RRP, 15% recycling and 80% reserve) (Harata et al., 2001a; Rizzoli and Betz, 2005; Suyama et al., 2007). These estimates are dramatically less than large, peripheral synapses that contain tens or even hundreds of thousands of synaptic vesicles (Harata et al., 2001a; Rowley et al., 2007; Schneggenburger et al., 1999). The relatively few vesicles that are available for central synapses
make these neurons particularly sensitive to changes in neurotransmitter loading, and therefore sensitive to changes in VACHT.

1.2 Populations of Cholinergic Neurons

Several populations of cholinergic neurons exist within the central and peripheral nervous system. These neurons form defined neuronal networks that contribute to many functions described in Section 1.3. As will be revealed, B6eGFPChAT mice exhibit global overexpression of VACHT. While this thesis cumulates at a discussion related to basal forebrain cholinergic neurons and their projections to the hippocampus (Chapter 5), our initial characterization of the mice explores cholinergic function related to a number of central and peripheral cholinergic pathways. In this section, the localization of those pathways will be briefly described.

1.2.1 Central Cholinergic Pathways

Initial anatomical work to define the central cholinergic pathways was accomplished with AChE histochemistry (Koelle, 1954). While effective at identifying cell somas and dendrites and axonal projections, this work lacked the ability to identify the polarity of fiber tracts. A pivotal advancement was made when it was observed that AChE enzyme would accumulate at the cell soma side of surgically transected cholinergic tracts, and would disappear from the opposite side (Lewis and Shute, 1967; Shute and Lewis, 1967). These techniques were applied to determine the start of cholinergic pathways and their axonal terminations. Subsequent work in the 1980s using retrograde transport of horseradish peroxidase confirmed the earlier pathways defined by Shute and Lewis (Lewis and Henderson, 1980). Since then, the cholinergic pathways have been refined by a number of groups. For consistency, the nomenclature proposed by Woolf will be used (Woolf, 1991; Woolf and Butcher, 2011).

1.2.1.1 Basal Forebrain Cholinergic Neurons

The basal forebrain complex includes several cholinergic nuclei (Fig. 1-2A). The complex includes, from rostral to caudal, the medial septum, vertical limb of the diagonal band of Broca, horizontal limb of the diagonal band of Broca, the nucleus basalis and the substantia innominata.
Fig 1-2. Central cholinergic pathways schematically represented on a sagittal section of a rodent brain. (A) Basal forebrain cholinergic neurons innervate the entire cortical mantle and hippocampus. (B) The mesopontine region provides diffuse innervation of the subcortical mass. Abbreviations: CPu, caudate putamen complex; hdb, horizontal diagonal band; ldt, laterodorsal tegmental nucleus; mh, medial habenula; ms, medial septum; nb, nucleus basalis; ppt, pedunculopontine tegmental nucleus; vdb, ventral diagonal band. (Adapted from Woolf, 1991)
The cholinergic basal forebrain provides extensive innervation to the entire neocortex and forebrain limbic structures. The medial septum and vertical limb of the diagonal band provides the majority of cholinergic innervation to the hippocampus, including the cornu ammonus (CA) 1-4 and the dentate gyrus (DG) (Woolf, 1991; Woolf and Butcher, 2011). This cholinergic projection from the septum to the hippocampus is commonly referred to as the septohippocampal pathway. In addition, the vertical limb of the diagonal band also projects to the thalamus, several cortices and the interpenduncular nucleus. The main outflow from the horizontal limb of the diagonal band is the olfactory bulbs, with additional projections to the amygdala, neocortex and entorhinal cortex (EC). Cholinergic neurons of the nucleus basalis and the substantia innominata provide vast innervation of the neocortex (Woolf, 1991; Woolf and Butcher, 2011). Cholinergic projections from the basal forebrain to its given target regions represent approximately 20-60% of the total terminals.

1.2.1.2 Striatal Cholinergic Interneurons

The striatum is generally subdivided into the dorsal striatum, consisting of the caudate nucleus-putamen complex and the ventral striatum consisting of the nucleus accumbens and the islands of Calleja (Woolf and Butcher, 2011) (Fig. 1-2B). The dorsal and ventral striatum each contain large, multipolar cholinergic interneurons, with a population of smaller cholinergic interneurons existing in the island of Calleja (Woolf and Butcher, 2011). These cholinergic interneurons, which represent approximately 2% of the total neuronal population of the striatum, receive dopaminergic input from the substantia nigra, which in turn is relayed back to the substantia nigra via gamma-aminobutyric acid (GABA) primary neurons in the striatum.

1.2.1.3 Other Cholinergic Populations

Two additional cholinergic populations are present in the mesopontine region, located in the laterodorsal tegmental nucleus and the pedunculopontine tegmental nucleus (Fig. 1-2B). Cholinergic neurons of the laterodorsal tegmental nucleus and the pedunculopontine tegmental nucleus diffusely project to the anterior, lateral and reticular nuclei of the thalamus, hypothalamus, basal forebrain and the spinal cord and brainstem (Woolf, 1991; Woolf and Butcher, 2011). In addition, these neurons provide the only known cholinergic input to midbrain dopaminergic neurons. Specifically, pedunculopontine tegmental cholinergic neurons target
dopaminergic neurons in the substantia nigra whereas laterodorsal tegmental cholinergic neurons target dopaminergic neurons in the ventral tegmental area (Oakman et al., 1995).

Finally, an intense population of cholinergic neurons in the epithalamus populate the medial habenula, which project through the fasiculus retroflexus fiber bundle and terminate at the interpeduncular nucleus (Woolf and Butcher, 2011).

1.2.2 Peripheral Cholinergic Pathways

Knowledge about the presence of chemoreceptors in the periphery system predates the identification of acetylcholine as a neurotransmitter. Early physiological studies showed that junctional regions of muscle were highly sensitive to chemical stimulants (Langley, 1907). Around this time, Loewi observed similar chemical simulation of the heart (Loewi and Navratil, 1926). Acetylcholine was subsequently shown to be responsible for the stimulation observed in earlier studies on these tissues (Buchthal and Lindhard, 1937; Dale and Dudley, 1929). Since these early studies, the role of cholinergic function in the PNS has been well established.

1.2.2.1 Somatic Nervous System

The somatic nervous system controls conscious voluntary control of skeletal muscle (Fleming, 2007). In this system, single neuron efferent pathways release acetylcholine to specialized post-junctional motor end plates (Fig. 1-3A). Acetylcholine signalling is primarily mediated using nicotinic acetylcholine receptors (nAChR), which result in excitatory contraction in skeletal muscle (Fleming, 2007).

1.2.2.2 Autonomous Nervous System

The autonomic nervous system regulates actions that are generally involuntary, and in this way differs from voluntary central and somatic nervous systems (Fleming, 2007). Autonomic motor neurons are found in the brainstem and the spinal cord. In contrast to the somatic and central systems, two neuron efferent connections are used where fibers extend beyond the CNS and synapse on autonomic ganglia (groups of neurons) with extensive synaptic connections to the periphery (Fleming, 2007). The autonomic nervous system consists of two portions, the parasympathetic and sympathetic nervous system, which can be classified by their anatomy and physiology (Fig. 1-3).
The parasympathetic system regulates the function of internal organs under resting conditions. Pre-ganglionic cholinergic fibers originate from the cranial spine and synapse with post-ganglionic cholinergic neurons (Fig. 1-3B). Post-ganglionic cholinergic fibers innervate cardiac and smooth muscle and salivary gland cells where muscarinic signalling provides inhibitory input (Fleming, 2007). The sympathetic system controls the up- and down-regulating of many homeostatic mechanisms. Perhaps the best characterized is the ‘flight-or-flight’ response that occurs following various stressors (Fleming, 2007). All pre-ganglionic fibers of the sympathetic system are cholinergic and activate nicotinic receptors on ganglionic peripheral neurons (Fig. 1-3C). Post-ganglionic fibers innervate cardiac and smooth muscle and salivary gland cells (via norepinephrine neurotransmission), sweat glands (via acetylcholine neurotransmission) and renal vascular smooth muscle (via dopaminergic neurotransmission). In addition, pre-ganglionic cholinergic fibers innervate the adrenal medulla, which stimulates the direct secretion of adrenaline (epinephrine) and to a lesser extent noradrenaline (norepinephrine) into the blood. Therefore, in the sympathetic division, acetylcholine regulates activity through both direct and indirect mechanisms.

1.3 Cholinergic Neurotransmission: General Functions

As mentioned in Section 1.2, the many cholinergic pathways in the central and peripheral nervous system have been implicated in a number of functional roles. In this thesis, particularly in Chapter 3, we have focused on evaluating central and peripheral functions that have previously been demonstrated to be affected by VACHT depletion. These include, metabolic and sleep regulation, motor function and anxiety-like behaviour. General descriptions of these functions along with the evidence that implicates VACHT in their modulation are provided below. In addition, the experimental techniques that are used to evaluated each function are introduced.

1.3.1 Metabolic and Sleep Regulation

The regulatory balance between the parasympathetic and sympathetic system regulates general metabolism. Specifically, sympathetic dominant metabolism leads to slow oxidation of energy, whereas parasympathetic dominant metabolism leads to fast oxidation of energy (Stralfors et al., 1984). That is because of sympathetic cholinergic stimulation of the adrenal gland leads to release of epinephrine and norepinephrine, which stimulates glycogen and lipid breakdown in the
Fig 1-3. Schematic representation of somatic and autonomic peripheral cholinergic pathways. (A) In the somatic nervous system, motor fibers release acetylcholine to specialized post-junctional motor end plates on skeletal muscle. (B-C) In the autonomic nervous system, preganglionic nerves release acetylcholine to peripheral ganglia. In the parasympathetic system (B) postganglionic nerves release acetylcholine to smooth muscle and cardiac tissue. In the sympathetic system (C), postganglionic nerves release either acetylcholine or norepinephrine to activate smooth muscle, cardiac tissue and sweat glands. Release of acetylcholine from the adrenal medulla results in secretion of norepinephrine and epinephrine to the blood. Abbreviations: ACh, acetylcholine; E, epinephrine; NE, norepinephrine. (Adapted from Fleming, 1997)
liver, adipose tissue and skeletal muscle (Bray, 1967). Direct studies measuring the modulation by acetylcholine of metabolic parameters for glycolysis and lipolysis in rodents are limited. In mice with striatal acetylcholine deficiency, the respiratory exchange ratio (RER) was significantly elevated compared to controls, indicating increased carbohydrate metabolism consistent with a withdrawal of sympathetic tone (Guzman et al., 2012). Attempts to augment cholinergic activity using AChE inhibitors or genetic manipulation do not appear to yield changes in energy metabolism (Androne et al., 2003; Kolisnyk et al., 2013).

Cholinergic neurotransmission is generally associated with a series of characteristic sleep changes, including decreased rapid eye movement (REM) latency and increased REM density (Sarter and Bruno, 1999; Vazquez and Baghdoyan, 2001). Mesopontine cholinergic neurons are believed to be the primary regulators of sleep-wake rhythms through their connections to the ventral tegmental area (Sarter and Bruno, 1999). Cholinergic neurons in these areas increase their firing rate during REM sleep and decrease during non-REM sleep (Sarter and Bruno, 1999). Epithalamic cholinergic signalling from the medial habenula may also contribute to sleep through the regulation of sleep duration, however this contribution is less established in the literature (Haun et al., 1992). Cholinergic signalling may also contribute to the cortical arousal associated with REM sleep, as acetylcholine release in the basal forebrain is highest during REM sleep, lower during quiet wakefulness, and lowest during non-REM sleep (Vazquez and Baghdoyan, 2001). In addition, cortical acetylcholine release is increased during wakefulness and REM sleep compared to non-REM sleep (Sarter and Bruno, 1999).

1.3.1.1 Experimental Models of Metabolic and Sleep Regulation

Calorimetry is based on the assumption that all cellular metabolic events ultimately result in heat (Ferrannini, 1988). Accurate measurement of heat production would then give information about the metabolic rate (i.e. direct calorimetry). However, in reality heat production is difficult to measure precisely and therefore indirect calorimetry has become the most commonly used method to measure metabolic rate. This method is based on measurements of oxygen (O$_2$) consumption, assumed to originate from oxidation of nutrients, and has proven to be a highly accurate estimate of energy expenditure (EE; heat). An animal inhales ambient air of a constant composition with the change in O$_2$ and carbon dioxide (CO$_2$) in expired air reflecting ongoing metabolic processes. Using specialized open gas exchange cages, maintained at thermoneutrality,
the volume of oxygen consumed (VO\textsubscript{2}) and carbon dioxide formed (VCO\textsubscript{2}) can be measured. The RER differs depending on the metabolic state (Ferrannini, 1988). During carbohydrate oxidation, six units of CO\textsubscript{2} and six units of O\textsubscript{2} are consumed, therefore RER is equal to one. During fat oxidation, 16 units of CO\textsubscript{2} and 23 units of O\textsubscript{2} are consumed, therefore RER will fall to 0.7. Using the RER, the calorific value can be derived and the heat generated by the animal can be calculated, reflecting the energy expended. In addition, activity and feeding sensors ensure measurements are collected when the animal is not eating or moving, and therefore the data relates to the resting metabolic rate. Using activity sensors within the cages, sleep-wake rhythms can also be established based on an algorithm that predicts sleep by detecting continuous bouts of inactivity (Pack et al., 2007).

1.3.2 Peripheral Motor Function

The striatum, and its connections to the substantia nigra collectively referred to as the nigrostriatal pathway, are primarily involved in the extrapyramidal motor system, providing control of involuntary reflexes and movement, and modulation of movement (coordination). These actions are typically modulated by dopaminergic signalling from the substantia nigra, which acts to increase motor activity. However striatal cholinergic interneurons are believed to inhibit the dopaminergic output of striatal principal neurons, thereby decreasing motor activity (Calabresi et al., 2000; Martins-Silva et al., 2011).

In addition to central cholinergic pathways, cholinergic motor neurons, originating from the sacral spine, synapse directly on skeletal muscle and modulate peripheral motor function. Augmentation of cholinergic activity at the neuromuscular junction (via AChE inhibition) has been shown to increase the amplitude and duration of end-plate potentials, causing muscle fibrillation (irregular action potentials) and fasciculation (visible spontaneous contractions) (Kordas, 1977; Sprouse and Baker, 1985). Insufficient cholinergic activity at the neuromuscular junction leads to myasthenia (Ohno et al., 2001; Prado et al., 2006).

1.3.2.1 Experimental Models of Peripheral Motor Function

The most standardized method of peripheral motor function in rodents is the open field (OF), an open arena where the animal is allowed to freely explore. Five-minutes of analysis are generally used to establish general motor activity, while analysis up to 2-hours in duration is useful for
more detailed analysis of motor activity and habituation (Crawley, 2007). Motor coordination and balance can be analyzed using the rotarod, a rotating cylinder where the rodent must continuously walk to keep from falling (Crawley, 2007). The rod gradually increases in speed during the trial, making the latency to fall a stringent measure of balance. Neuromuscular abnormalities can also be detected using hanging wire and grip strength paradigms. In the hanging wire test, balance and grip strength are measured by suspending the rodent upside-down from a wire mesh. The latency to fall is an indicator of neuromuscular strength. In a similar assay, the grip strength test involves lowering the rodent onto a tension gauge, which it grips with its forepaws. The mouse is then pulled from the tension gauge along the horizontal axis and peak tension is recorded as a measure of neuromuscular strength (Crawley, 2007).

1.3.3 Anxiety-like Behaviour

The majority of literature utilizing hippocampal lesions predicts regional dissociations within the hippocampus, highlighting a ventral-dependant preference for anxiety-like behaviour. However, a specific role for cholinergic neurons appears to exist throughout the hippocampus, and may be dependent on the type of anxiety-like behaviour being measured. In this thesis, we have focused on environmental anxiety, resulting from conflict generated by the animal’s surroundings. Using targeted micro-infusions, the administration of AChE inhibitors into the dorsal or ventral hippocampus has been shown to reduce environmental anxiety (Engin and Treit, 2007). In contrast, the application of nicotine, a nAChR agonist, in the dorsal hippocampus has no effect (Engin and Treit, 2007; File et al., 1998). These results suggest that environmentally stimulated anxiety-like behaviour may be mediated through both dorsal and ventral muscarinic signalling.

1.3.3.1 Experimental Models of Anxiety-like Behaviour

The measurements of anxiety in rodents are often considered ‘endophenotypes’ as there is no definite method to determine the rodent’s emotional state (Crawley, 2007). Anxiety-like behaviour can be established by observing the response of rodents to environments, social interactions and events. The most common tests used to study environmentally stimulated anxiety-like behaviour in rodents are the elevated plus maze (EPM), the OF and the dark-light box (DLB) (Barnett, 1958; Crawley and Goodwin, 1980; Handley and Mithani, 1984). These
Fig. 1-4. Elevated plus maze (EPM) apparatus. The maze consists of four arms that radiate from a central square, in the shape of a “plus”. Two arms are closed (enclosed with 40.6 cm tall opaque walls) and two arms are open. Mice are placed into the maze and allowed to freely explore. Anxiety-like behaviour is interpreted by the amount of time spent in the center, closed and open arms.
assays generate unconditioned, natural conflicts in the rodents and titrates the tendency of mice to explore novel environments against the aversive properties of brightly lit, open fields. The most widely used measure is the EPM (Fig. 1-4). The EPM is an apparatus in the shape of a “plus”, with four arms radiating from a central square. Two of the arms are enclosed with walls (closed arms) while two of the arms are open platforms (open arms). More time spent in the open arms is considered indicative of lower levels of anxiety (Belzung and Griebel, 2001). In the OF (Fig. 1-5), anxiety is measured through the evaluation of thigmotaxis, or the tendency for the animal to stay within physical contact with a fixed structure of the environment (Crawley, 2007). In this assay, higher proportion of time spent in the center of a square field (i.e. not in contact with the periphery) is indicative of less anxiety. Finally, in the DLB (Fig. 1-6), the square arena is separated into an enclosed, dark region and an open, lit region that are divided by a small opening. In this assay, the conflict is primarily based on the lit environment, as the animal can still perform thigmotactic exploration of the open, lit region. An increased proportion of time spent in the open, lit region is therefore indicative of lesser anxiety (Crawley, 2007). The ability of the EPM, OF and DLB to detect anxiety-like behaviour has been demonstrated with a number of anxiolytic or anxiogenic pharmacological agents and transgenic manipulations (Belzung and Griebel, 2001; Crawley, 1985; Lister, 1987; Prut and Belzung, 2003).

1.4 Cholinergic Neurotransmission: Spatial Cognition and Adult Neurogenesis

The hippocampus is innervated by cholinergic projections from the septum (via the septohippocampal pathway). This pathway provides the primary source of acetylcholine for hippocampal function. Abnormalities in cholinergic activity, associated with both normal and pathological (i.e. AD) aging profoundly impact hippocampal information processing (Bartus et al., 1982; Bartus, 2000). More recently, hippocampal-dependant spatial cognition has been associated with adult neurogenesis (Garthe and Kempermann, 2013), a process that is also regulated by septohippocampal acetylcholine (Cooper-Kuhn et al., 2004; Mohapel et al., 2005). In this thesis, specifically in Chapter 4, the spatial memory performance of B6eGFPChAT mice was evaluated. A description of the concepts, along with experimental and analytical techniques used to assess spatial cognition and adult neurogenesis is provided.
Fig. 1-5. Open field (OF) apparatus. The square field is 42 cm x 42 cm and bound by 30 cm tall walls. Mice are placed into the field and allowed to freely explore. Locomotion can be measured as horizontal or vertical activity. Anxiety-like behaviour is interpreted by measuring the time spent in the center (marked in green) compared to the periphery (thigmotaxis).
Fig. 1-6. Dark-light box (DLB) apparatus. The open field (Fig. 1-5) is separated into two spaces with an opaque divider, with a small passage. Mice are placed into the field and allowed to freely explore. Locomotion can be measured as horizontal or vertical activity. Anxiety-like behaviour is interpreted by the amount of time spent in, and transitions between, the enclosed dark and the open lit regions.
1.4.1 Spatial Cognition

Spatial cognition relates to the encoding and retrieval of the location of objects in space. This function is achieved by structuring spatial information into a series of coordinates. The elements that generate spatial structures are grid cells within the dorsal caudal medial EC and place cells within the DG and CA regions of the hippocampus. A grid cell is active at several locations within an environment, such that the firing pattern forms a triangular grid (Hafting et al., 2005). The orientation of the grid relative to the environment is fixed in relation to polarizing visual cues in the arena. Grid cells take into account the speed at which the animal moves through the environment, in order to maintain the proper firing nodes. It is thought that grid cells provide the Euclidean distance and direction metrics that map to hippocampal place cells (O'Keefe and Dostrovsky, 1971). A place cell is active at a single location within an environment. The way that spatial coordinates are navigated involves the use of two reference systems. Egocentric spatial transformations involve self-to-object encoding where information about one object is related to the user's position and orientation in space. This approach stands in contrast to allocentric spatial transformation, and involves encoding information about one object based on the relative position of other objects. The effective mapping of spatial information onto place and grid cells is thought to engage the hippocampus, and is dependent on adult neurogenesis.

1.4.2 Adult Neurogenesis

In the 1960s, Joseph Altman and Gopal Das challenged the central assumption that neural stem cells are depleted in the perinatal brain and that neurogenesis ceases during adulthood. Through this work, autoradiography with $[^{3}\text{H} \text{-thymidine}}$, which incorporates into newly dividing deoxyribonucleic acid (DNA), was used to identify cell proliferation in the brain (Altman and Das, 1965). However, with the lack of appropriate markers, the neuronal identity of these cells could not be concluded. In the 1980s, ultrastructural analysis of $[^{3}\text{H} \text{-thymidine}}$ labelled cells suggested that they were in fact neurons (Kaplan and Bell, 1984; Kaplan and Hinds, 1977), however this work was met with considerable skepticism primarily because the cell type could not be conclusively determined. In the late 1980s and early 1990s, the introduction of the thymidine analogue 5-bromo-2-deoxyuridine (BrdU), which could be detected immunocytochemically, was coupled to novel cell type-specific markers for immunohistochemical identification of the phenotype of newly generated cells (Cameron et al.,
These developments replicated the earlier findings proposed three decades earlier, and finally established the process of mammalian adult neurogenesis.

The presence of functional progenitor cells in the adult human hippocampus has also been demonstrated using in vitro cultures (Roy et al., 2000). Adult neurogenesis has been confirmed in human subjects with squamous cell carcinoma who consented to BrdU administration (Eriksson et al., 1998). Recently, the generation of adult human hippocampal cells was again confirmed through carbon dating of $^{14}$C derived from above ground nuclear testing during the Cold War (Spalding et al., 2013). Furthermore, the rate of human adult neurogenesis matches that of middle-aged mice (Spalding et al., 2013). Human adult neurogenesis may therefore be required for similar functionalities as described in rodents, including the mediation of cognition.

### 1.4.2.1 Functions of Adult Neurogenesis

One of the best characterized roles of adult neurogenesis relates to its involvement in memory and learning. Numerous studies have correlated changes in hippocampal adult neurogenesis (mainly cell proliferation) with altered cognition using physical activity (Van Praag et al., 1999; Van Praag et al., 2005), enriched environments (Kempermann et al., 2002), and aging (Driscoll et al., 2006; Kuhn et al., 1996) as experimental paradigms. It has yet to be determined whether hippocampal neurogenesis is a causal factor for the observed changes in cognition, however unique electrophysiological properties of newborn dentate neurons predicts that they may play a transient, yet important role in learning (Wang et al., 2000). Information from sensory cortical areas converges on the trisynaptic circuit of the hippocampus (Fig. 1-7). At this point, the information is passed through one of two pathways. In the first route, information is passed from the EC directly to the CA3, CA1 and returned to the EC via the subiculum (Deng et al., 2010). In the second route, information from the EC is first passed to the DG via the perforant path (Deng et al., 2010). The DG is believed to compress and orthogonalize the high amount of cortical information, which is subsequently passed to the CA3 (Clelland et al., 2009; Garthe and Kempermann, 2013).

For this, the DG exercises pattern recognition of the EC input patterns. When necessary, pattern separation is used to make similar input patterns into more distinct output patterns (creating new memory traces), and pattern completion is used to associate input patterns to closely associated output patterns (auto-associative memory) (Garthe and Kempermann, 2013). In spatial memory,
Fig. 1-7. Neural circuitry of the hippocampus. Information from the entorhinal cortex (EC) is processed via the perforant path either directly to the pyramidal cells of the cornu ammonis 3 (CA3), or through the granule cells of the dentate gyrus (DG) which project mossy fibers to the CA3. Schaffer collaterals relay the information to the cornu ammonis 1 (CA1) which project back to the EC. Abbreviations: GCL, granule cell layer; ML, molecular layer. (Adapted from Deng et al., 2010)
these output patterns relate to CA place cells with firing patterns highly correlated to physical positions within an arena. Place cells are generated in new environments, and these cells provide the basis for cognitive maps in the hippocampus. While DG input to the CA regions does not govern the spatial specificity of place cells per se, DG input is necessary to establish place cell fields with the behavioural context itself (i.e. through pattern recognition). In other words, DG activity is critical to the efficient spatial navigation within an arena. The renewing population of adult hippocampal neurons is thought to be directly related to this process (Clelland et al., 2009).

1.4.2.2 Localization of Adult Neurogenesis

Adult neurogenesis has been shown to occur in a variety of species, in the hippocampus (Altman and Das, 1965; Eriksson et al., 1998; Gould et al., 1998; Kaplan and Bell, 1984; Kaplan and Hinds, 1977), and the lateral ventricles (Altman and Das, 1966; Kornack and Rakic, 2001b). Other regions of the brain have been suggested to be neurogenic, including the CA1 of the hippocampus (Rietze et al., 2000), substantia nigra (Zhao et al., 2003), amygdala (Bernier et al., 2002), striatum (Bédard et al., 2002; Bédard et al., 2006), hypothalamus (Kokoeva et al., 2005), and in the neocortex (Gould et al., 1999b). However, these additional regions remain controversial (Feliciano and Bordey, 2013; Kornack and Rakic, 2001a), occurring at infinitesimally small rates, with very poor survival, or only under certain physiological conditions. Given the scope of this thesis, only adult neurogenesis within the hippocampus will be discussed.

1.4.2.3 Development of Adult-born Hippocampal Neurons

In the hippocampus, newborn cells are generated from adult neural stem cells (Gross, 2000; Suh et al., 2007). Neural stem cells exhibit potential for self-renewal and differentiation into neural cells (Gage et al., 1998). The development of neural stem cells into mature granule neurons can be traced with unique morphological and molecular markers (Fig. 1-8). Type 1 neural stem cells have both radial and horizontal processes, express nestin, glial fibrillary acidic protein and the Sry-related HMG box transcription factor (Brazeal et al., 2005; Zhao et al., 2008). Type-1 cells divide to produce intermediate Type 2a/b transit amplifying progenitor cells (Steiner et al., 2006). Type-2a/b progenitor cells divide rapidly and generate Type-3 neuroblasts cells with early
Fig. 1-8. Neurogenesis in the rodent hippocampus. Type 1 neural stem cells give rise to type 2 neural progenitor cells, identified by their distinct morphology and molecular markers (see text). By ~1 week, immature neurons project doublecortin-positive dendritic projections and receive GABA input. A transition from GABA excitability to GABA inhibition occurs by ~2.5 weeks when dendrites project through to the molecular layer (ML). By ~4-8 weeks, newborn cells become indistinguishable from the existing neuronal network. Abbreviations: CA1/3, cornu ammonis 1/3; GCL, granule cell layer; ML, molecular layer. (Adapted from Zhao et al., 2008)
neuronal features (Steiner et al., 2006). Type-3 neuroblasts express the immature neuron marker (doublecortin) DCX in their dendrites, which by approximately 2 weeks of age have extended through the granule cell layer to the molecular layer. Additional features of these immature neurons are enhanced glutamatergic and GABA excitability due to initially high intracellular chloride concentrations, leading to membrane depolarization (Ge et al., 2007). These committed Type-3 neuroblasts generate immature granule neurons, and those that survive through the activity-dependant survival period integrate into the granule cell layer within 4 weeks (Gu et al., 2012; Kempermann et al., 2004). As granule cells mature, GABA inputs become hyperpolarizing and inhibitory (Zhao et al., 2008). Between 4-8 weeks of age, the newborn neurons become both morphologically and physiologically indistinguishable from the existing neuronal network.

1.4.2.4 Cholinergic Contribution to Adult Neurogenesis

Despite neurogenesis persisting into adulthood, continued aging has been associated with dramatic reductions the number of newborn neurons generated in the hippocampus. Studies in rodents that have quantified the number of proliferating cells, using the thymidine analogue BrdU, have revealed that the most dramatic age-related decline is evident in the number of proliferating cells formed at the subgranular zone (SGZ). Following initially high levels of proliferation during development and during adolescence, the number of proliferating neurons in the SGZ is significantly decreased by 6 months of age, with additional decline occurring to 24-months of age (Bondolfi et al., 2004; Gil-Mohapel et al., 2013; Kempermann et al., 1998; Kuhn et al., 1996; Nacher et al., 2003). The sharp reduction in proliferating cells is thought to represent a true decrease in the number of newborn cells and not changes in cell cycle kinetics (Luo et al., 2006). However, the mechanism for reduced proliferation during aging is not fully understood. There have been reports that neural progenitor cells, isolated from young and old rodents and cultured in vitro, generate comparable numbers of neurospheres (Jin et al., 2003), suggesting that the proliferative changes observed in vivo are related to decreased growth factors and hormones within the neurogenic milieu, as opposed to factors intrinsic to the cell (Villeda et al., 2011). Aged progenitor cells can respond to a variety of stimuli, exhibiting neurogenic levels seen in young adults (Cameron and McKay, 1999; Kempermann et al., 1998; Kempermann et al., 2002; Nilsson et al., 1999; Van Praag et al., 2005). These data suggest that given the proper environment, the impaired proliferative capacity of aged progenitors can be restored.
The depletion of cholinergic input to the neurogenic milieu during the aging process may also contribute to these deficits through dysregulation of cell proliferation (Cooper-Kuhn et al., 2004; Kaneko et al., 2006; Mohapel et al., 2005; Schliebs and Arendt, 2006). In addition, cholinergic input may support an environment permissive of dendritic expansion in newborn neurons (Campbell et al., 2010). An expansion of the dendritic network may enhance neuronal connectivity and supports the beneficial role of newborn neurons in the processes involved in efficient spatial cognition.

1.4.3 Experimental Models of Spatial Cognition

One of the most established laboratory techniques to study spatial navigation is the Morris water maze (MWM) (Morris, 1984). In this test, the rodent is trained through successive trials to swim through an open circular pool to find an escape platform. Studies have revealed roles for numerous brain structures, and likely their integrated coordination allows for successful spatial navigation to the target (Reviewed by Garthe and Kemperman, 2013). It is for this reason also that general performance in the MWM does not rely exclusively on the hippocampus. The hippocampus is, however, believed to integrate egocentric, route-based knowledge into allocentric cognitive maps that ultimately facilitates navigation by spatially focusing search locations (Garthe and Kempermann, 2013). The specificity of the cognitive map is based on the detail of the egocentric route based coverage, and the ability of the hippocampus to integrate this information into the cognitive map, the contribution of the latter increasing with repeated MWM trials.

The specific MWM protocol that is best suited to evaluate the generation of cognitive maps is the spatial memory protocol (Vorhees and Williams, 2006). In this rendition, the animal is trained to use multiple cues external to the maze to triangulate its position and locate the platform that is hidden from direct sight (located 1 cm below the surface of the water) (Fig. 1-9). To encourage the use of allocentric navigation, during each trial of each training day, the start position is pseudo-randomized to one of four different entry positions around the maze. This prevents the animal from using egocentric navigation (i.e. always turning left or right) and provides a specific evaluation of hippocampal-dependant spatial memory in the subject.

Classical MWM parameters, which typically include escape latency and distance to the escape platform, are ideal for the analysis of egocentric navigations. However, as the hippocampal
Fig. 1-9. Morris water maze apparatus. Mice are placed into the pool of water at one of four insertion points located 90° from each other and trained over four days of four trials per day to locate an invisible platform located 1cm below the surface of the water. Fixed, visual cues located around the exterior of the maze apparatus provide visual stimuli for navigation. Following training, mice are exposed to a single probe trial where the invisible platform is removed.
contribution to MWM performance appears to be well suited to the formation of allocentric representations, this may explain the discrepancies in the literature relating impaired neurogenesis to MWM performance. Recent studies have evaluated the study of spatial search strategies in the analysis of MWM performance (essentially quantifying the specificity of the allocentric cognitive map during the trial) (Garthe et al., 2009; Gil-Mohapel et al., 2013). When search strategy analysis is coupled to the spatial memory MWM protocol, identifying the contribution of hippocampal neurogenesis to MWM learning is greatly enhanced (Garthe et al., 2009; Garthe and Kempermann, 2013) and has been recently correlated (Gil-Mohapel et al., 2013).

### 1.5 Utility of Mouse Models with Modified VACChT Expression

#### 1.5.1 Mouse Models of VACChT Depletion

The last eight years has seen the development of numerous models of VACChT-deficiency. In the earliest work, VACChT was eliminated from the CNS and PNS (de Castro et al., 2009a). While this work demonstrated the dependence of acetylcholine release and the generation of MEPPs on VACChT mRNA and protein expression, these mice only survived for minutes after birth, likely due to insufficient neuromuscular tone at the diaphragm. Subsequent use of heterozygous VACChT knockout mice, with a 50% decrease in VACChT mRNA levels as controls did not reveal a behavioural phenotype, likely due to presynaptic compensation through increased CHT expression (de Castro et al., 2009a).

Subsequent models mutated the VACChT gene through the insertion of the neo-tk selection cassette at the 5’-untranslated region of VACChT (Prado et al., 2006) or a region upstream of the 5’-untranslated region (Martins-Silva et al., 2011) to suppress VACChT expression without abolishing it completely. From this work, it was found that >50% reductions of peripheral VACChT expression significantly impaired neuromuscular function (as measured by grip strength, hanging wire and rotarod) compared to controls (Martins-Silva et al., 2011; Prado et al., 2006). On the other hand, even modest reductions of VACChT expression were shown to affect multiple cognitive domains, including motor learning, object and social recognition (de Castro et al., 2009b; Prado et al., 2006) as well as induce locomotor hyperactivity (Martins-Silva et al., 2011). In these cases, spatial memory was spared (de Castro et al., 2009b).
The specific contribution of cholinergic populations, and their regulation by levels of VACHT, was significantly enhanced by two transgenic VACHT-depletion models, with tissue specific deletion driven by the D2 (VACHT-D2KO) and Six3 (VACHT-Six3KO) promoters. In VACHT-D2KO mice, VACHT expression is virtually eliminated in the striatum. These mice exhibit normal neuromuscular function (including grip strength, hanging wire, rotarod), spontaneous locomotor activity and cognition (Guzman et al., 2011). VACHT-D2KO mice do, however, show altered energy homeostasis (increased RER, suggesting higher carbohydrate utilization) and variations in sleep-wake cycles (increased periods of wakefulness) (Guzman et al., 2012). VACHT-Six3KO mice exhibit selective depletion of VACHT in both the basal forebrain (85% of controls) and striatum (58% of controls) (Martyn et al., 2012). The specific contribution of basal forebrain VACHT deletion leads to locomotor hyperactivity and impaired hippocampal-dependant spatial memory (Martyn et al., 2012).

Collectively, these findings highlight a number of roles for VACHT-regulated cholinergic neurotransmission. First, central cholinergic pathways appear to be more sensitive to changes in VACHT expression than peripheral pathways. Second, tissue-specific VACHT-depletion models predict a role in striatal cholinergic neurons in the regulation of homeostasis and the sleep-wake cycle, while basal forebrain cholinergic neurons are involved in the modulation of spontaneous locomotion and hippocampal-dependant spatial memory.

The considerable work on models of VACHT depletion continue to offer insight on neurodegenerative mechanisms that occur during both normal and pathological aging. These studies have identified VACHT as a target to improve many behavioural impairments. What was yet to be developed, prior to the work completed in this thesis, was a viable animal model of VACHT overexpression.

1.5.2 Mouse Models of VACHT Overexpression

Prior to the work composing this thesis, models of VACHT overexpression were limited to in vitro cultures of dissociated cells genetically modified to enhance VACHT expression (Song et al., 1997). Through these studies, the potential of VACHT to regulate quantal size was first described; however, it remained to be determined whether the affect of VACHT overexpression would be maintained in vivo. At the early stages of my graduate studies, I discovered that transgenic mice, originally developed as enhanced green fluorescent protein (eGFP)-ChAT
reporter lines, exhibit potential for VChT overexpression. This model (Nagy and Aubert, 2012) (see Chapter 2) utilized a bacterial artificial chromosome (BAC) transgene that contained the cholinergic gene locus coding region in which the eGFP coding region was inserted at the ChAT start codon. For its original purposes, the BAC would drive eGFP expression under the ChAT promoter and could be used to identify cholinergic neurons. However, an unmodified region of the inserted BAC contained the entire VChT coding region in addition to its predicted promoter. As a result, while the ChAT-eGFP-containing BAC transgene faithfully reports cholinergic neurons, it also exhibits the potential to express VChT. Furthermore, given that BACs typically integrate into the host genomes in multiple, head-to-tail concatemeric copies, VChT was predicted to be overexpressed. However, this had yet to be determined.

Given that the previous reports of VChT deficiency led to decreased cholinergic tone, I became interested in the use of B6eGFPChAT mice to study VChT overexpression in vivo and determine whether this would lead to improvements in cholinergic tone and related functions.

### 1.6 Hypothesis and Specific Aims of the Thesis

#### 1.6.1 Hypothesis

I propose that B6eGFPChAT mice exhibit enhanced VChT expression that leads to enhanced central and peripheral cholinergic tone, increasing cholinergic-related neuroplasticity and functions, including hippocampal neurogenesis and hippocampal-dependant spatial memory performance during aging.

#### 1.6.2 Specific Aims

To test this hypothesis, the following specific aims were addressed:

1. Characterize the gene and protein expression of VChT and related presynaptic cholinergic proteins and evaluate their affect on acetylcholine release from terminals in the hippocampus (Chapter 2),
2. Establish the general behavioural phenotype, focusing on the influence of central and peripheral cholinergic pathways (Chapter 3), and
3. Evaluate the specific changes in hippocampal-dependant spatial memory and hippocampal neurogenesis during aging of B6eGFPChAT mice (Chapter 4).
Chapter 2
Overexpression of the vesicular acetylcholine transporter increased acetylcholine release in the hippocampus

Contents of this chapter have been published:


P.M.N. led and conducted all experiments and drafted the manuscript. I.A. provided materials, analysis tools and experimental design as well as manuscript revision.
2.1 Abstract

Cholinergic neurotransmission in the hippocampus is involved in cognitive functions, including learning and memory. Strategies to enhance septohippocampal cholinergic neurotransmission may therefore be of therapeutic value to limit cognitive decline during cholinergic dysfunction. In addition to current strategies being developed, such as the use of AChE inhibitors, enhancing acetylcholine release may be critical for optimal cholinergic neurotransmission. VAChT activity limits the rate of formation of the readily releasable acetylcholine pool. As such, we sought to determine the influence of increased VAChT expression on the septohippocampal cholinergic system. To do this, we used the B6eGFPChAT congenic mouse, which we show contains multiple gene copies of VAChT. In this transgenic mouse, the increased VAChT gene copy number led to an increase in VAChT gene expression in the septum and a corresponding enhancement of VAChT protein in the hippocampal formation. VAChT overexpression enhanced the release of acetylcholine from \textit{ex vivo} hippocampal slices. From these findings, we conclude that VAChT overexpression is sufficient to enhance acetylcholine release in the hippocampal formation. It remains to be established whether, in cases of cholinergic deficits, increasing VAChT expression would re-establish adequate levels of cholinergic neurotransmission, thereby providing a valid therapeutic target.

2.2 Introduction

Cholinergic neurons synthesize and release the neurotransmitter acetylcholine. Cholinergic neurons of the medial septal area of the basal forebrain provide a major source of innervation to the hippocampal formation. Cholinergic neurotransmission through the septohippocampal pathway has been reported to affect learning, memory, attention and sleep (Drever et al., 2011; Sarter and Parikh, 2005). However, the mechanisms that contribute to the regulation of cholinergic activity are not fully elucidated, in particular with regards to the role of the VAChT.

Appropriate synthesis, packaging and release of acetylcholine is critical to the maintenance of cholinergic neurotransmission. Choline, a precursor to acetylcholine, is sequestered in the presynaptic terminal by CHT and transacetylated by ChAT (Sarter and Parikh, 2005). In mammalian systems, ChAT is often in kinetic excess (Brandon et al., 2004; Hersh, 1982) and as such, the supply of choline via CHT is considered to be rate limiting to acetylcholine synthesis.
VAChT packages newly formed acetylcholine into synaptic vesicles to prepare its release to the synaptic cleft. VAChT activity may affect the generation of the readily releasable acetylcholine pool. Specifically, in neurons that possess small releasable pools of synaptic vesicles, including central cholinergic neurons, the rate of filling recycling vesicles may be directly influenced by the level of VAChT (Prado et al., 2002).

Studies using animal models of VAChT deficiency and neuronal in vitro cell culture have revealed that the quantity and localization of VAChT provides stringent control on acetylcholine release (de Castro et al., 2009b; Martins-Silva et al., 2011; Prado et al., 2006; Song et al., 1997). VAChT-knockdown mice with approximately one-half of the wild-type VAChT expression exhibit decreased amplitude and frequency of MEPPs as a result of decreased acetylcholine packaging (Prado et al., 2006). These mice exhibit both general myasthenia and behavioural deficits in object recognition and social behaviour consistent with decreased cholinergic function (de Castro et al., 2009b; Prado et al., 2006). Early work involving the overexpression of rat VAChT cDNA in Xenopus spinal cord neurons demonstrates VAChT-dependent increases in synaptic activity (Song et al., 1997) and provides seminal findings implicating VAChT as a possible target to enhance cholinergic function. Despite the promise of these earlier findings, a comprehensive molecular analysis of the in vivo effects of VAChT overexpression has not been investigated in mammalian systems. We therefore sought to determine the effect of increased VAChT expression on acetylcholine release in cholinergic neurons of the mouse brain.

Briefly, we used a B6eGFPChAT congenic mouse that, as we discovered, exhibits VAChT overexpression by virtue of its ChAT-eGFP genetic modification. Using this model, we provide in vivo experimental evidence that increasing septal VAChT expression leads to increased VAChT protein levels while ChAT, CHT and AChE expression and activity are unchanged. Furthermore, we reveal that acetylcholine release is increased to the hippocampus in the B6eGFPChAT congenic mouse. We conclude that VAChT overexpression in septal cholinergic neurons is sufficient to enhance acetylcholine release to the hippocampus. Our study supports the notion that increasing VAChT expression may serve as a therapeutic approach to enhance cholinergic neurotransmission.
2.3 Experimental Procedures

2.3.1 Animals and Brain Samples

Male and female mice from the congenic strain B6.Cg-Tg(RP23-268L19-EGFP)2Mik/J (B6eGFPChAT) and the appropriate age- and sex-matched C57BL/6J (B6) control mice were obtained from Jackson Laboratories (Bar Harbour, ME). Presence of the transgene was confirmed using conventional polymerase chain reaction (PCR) and primers as previously described (Tallini et al., 2006), and by the observed expression of eGFP. All animal protocols were approved by the Animal Care Committee of Sunnybrook Health Sciences Centre, and experiments were performed according to the guidelines set by the Canadian Council on Animal Care and the Animals for Research Act of Ontario.

VAChT-knockout (VAChT-KO) striatal brain homogenates from adult VAChT\(^{D2cre-flox/flox}\) mice (Guzman et al., 2011) were generously provided by Dr. Vania Prado. These samples were used as negative controls for Western blot experiments.

2.3.2 Quantitative PCR (qPCR)

B6eGFPChAT and B6 (13 weeks old, n=4 per group) genomic DNA (gDNA) was extracted from 1.2 cm fragments of mouse tail using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma, Oakville, ON) following the manufacturer’s instructions. Primers for the target genes ChAT (forward primer, CCT CAT CTC CAA GGA TGG AA; reverse primer, CAG GTC CCG CTC TCT AAC AG), CHT (forward primer, CTC TCT TGA CGG GCT AGT GG; reverse primer, GCT CAG TCA CAA CCC CAA AT), VAChT (forward primer, CAG CTT TGG AAG CCT AGT GG; reverse primer, AGG AGT AGG AGT GCG TCG AA) and for the reference gene β-actin (forward primer, TTA GGT ATG GAA TCC TGT GG; reverse primer, TGT TGG CAT AGA GGT CTT TAC G) were commercially generated (Invitrogen, Burlington, ON). Target specificity was first confirmed through \textit{in silico} PCR against the B6 mouse genome (Genome Bioinformatics Group, University of California Santa Cruz) followed by visualizing amplification products using conventional PCR on a 3% high-resolution agarose gel. All applications of B6eGFPChAT and B6 gDNA input for qPCR were performed in triplicate. QuantiTect SYBR Green PCR mastermix (Qiagen, Mississauga, ON) was used with 1 μL forward and reverse primers (0.5 μM each). Eighteen microlitres of master-mix was applied to
glass capillaries and a 2 μl volume of gDNA (either 0.016, 0.08, 0.4, 1, 2, or 10 ng) was added as PCR template. Capillaries were closed, centrifuged and placed into a cycling rotor. A four-step experimental run protocol was used: (i) denaturation and Taq activation program (15 min at 95°C); (ii) amplification and quantification program repeated 50 times (15 s at 94°C; 20 s at 55°C; 15 s at 72°C); (iii) melting curve program (15 s at 95°C; 1 min at 55°C; 15 s at 95°C); (iv) cooling program down to 4°C. Fluorescence crossing point (CP) was defined as the point at which the fluorescence rises appreciably above the background fluorescence and was determined using the Second Derivate Maximum Method by the LightCycler Software 3.5 (Roche Diagnostics, Laval, QC). Relative expression software tool was used to analyze the amplification results (Pfaffl et al., 2002). The pair wise fixed reallocation randomization test was used to test significance between the B6eGFPChAT and B6 CP values.

2.3.3 Quantitative RT-PCR (qRT-PCR)

B6eGFPChAT and B6 mice (13 weeks old, n=3 per group) were deeply anesthetised, sacrificed and the brains were isolated. The septal areas were rapidly dissected on ice and flash frozen in liquid nitrogen. Tissue samples were homogenized using sterile blunt fill syringes and a QiaShredder (Qiagen) as per the manufacturer’s instructions. Total ribonucleic acid (RNA) was extracted using the RNeasy Mini Kit (Qiagen), quantified using photospectrometry and assessed for quality using an Agilent 2100 Bioanalyzer (Aligent, Mississauga, ON). 500 ng of total RNA was reverse transcribed using the Superscript VILO cDNA synthesis kit (Invitrogen). cDNA was subsequently subjected to qRT-PCR on an ABI Prism 7000 real-time PCR system (Applied Biosystems, Carlsbad, CA) using FAST SYBR green PCR master mix (Invitrogen) and QuantiTect primer assays for VACHT, ChAT and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Qiagen). For each experiment, a non-cDNA template reaction was used as a negative control. The specificity of the PCRs was also confirmed with melting curve analysis and by verification of single amplicons by 3% agarose gel electrophoresis. B6eGFPChAT and B6 target CP values were normalized using GAPDH control expression.

2.3.4 Semi-quantitative RT-PCR (RT-PCR)

Total RNA was extracted as previously described. 500 ng of total RNA was reverse transcribed using the Superscript VILO cDNA synthesis kit (Invitrogen) and diluted 10X in H2O prior to
RT-PCR. 3 µL of diluted cDNA was used in PCR reactions with primers targeting ChAT (forward primer, CCT GCC AGT CAA CTC TAG CC; reverse primer, CTC GAT CAT GTC CAG GGA GT), VACHT (forward primer, TTG ATC GCA TGA GCT ACG AC; reverse primer, GAA ACG CAA GGG GAA TGT TA), CHT (forward primer, GTG GTC TAG CTT GGG CTC AG; reverse primer, AGC TGC CAG GAA GGA CAG TA), AChE (forward primer, ATG CCA TGA GTG CAG TGG TA; reverse primer, TGA GCA ATT TGG GGA GAA AG) and GAPDH (forward primer, ACT CCA CTC ACG GCA AAT TC; reverse primer, CAC ATT GGG GGT AGG AAC AC). Cycle times were 30 seconds at 94°C, 30 seconds at 57.1°C (ChAT amplification), 56.1°C (VACHT, CHT, and GAPDH amplification) or 56.3°C (AChE amplification) and 30 seconds at 72°C. The number of cycles was optimized for each cDNA in preliminary experiments to determine exponential amplification range. PCR products were electrophoresed on a 1.25% agarose gel, stained with ethidium bromide and visualized under ultraviolet light. Signal intensities were quantified using GeneTools software (Syngene, Frederick, MD).

2.3.5 Immunohistochemistry

B6eGFPChAT and B6 mice were concurrently anaesthetized with a mixture of ketamine (75 mg/kg) and xylazine (10 mg/kg). They were then perfused intracardially with saline, followed by fixation with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed, post-fixed overnight and equilibrated in 30% sucrose. Coronal sections from each genotype were simultaneously cut at 30 µm and collected in 96-well plates filled with cryoprotectant solution (50 mM phosphate buffer; 25% (v/v) glycerin; 30% (v/v) ethylene glycol; pH 7.4).

Four serial sections from B6eGFPChAT and B6 mouse brains (13 weeks old, n=4 per group) were processed in parallel, using the same conditions, solutions and incubation periods. Sections were rinsed with 0.1 M phosphate-buffered saline (PBS, pH 7.4), treated in a solution of 0.6% aqueous hydrogen peroxide for 15 minutes, rinsed with PBS, and incubated for 30 minutes at room temperature in a blocking solution of 5% normal donkey serum and 0.3% Triton X-100 in PBS (this solution was used to dilute all antibodies). Sections were incubated with goat anti-ChAT (AB144P; Millipore, Temecula, CA) or rabbit anti-VACHT (generously provided by Dr. Vania Prado, Robarts Research Institute, London, Ontario) for 24 or 72 hours at 4°C respectively. Control sections were incubated in the blocking solution lacking primary
antibodies. All sections were rinsed in PBS and incubated for 2 hours at room temperature in a 1:200 solution of secondary biotinylated donkey anti-species antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Sections were rinsed in PBS and incubated for 1 hour at room temperature in Vectastain Elite avidin-biotin complex (PK-6100; Vector Laboratories, Burlingame, CA) and incubated in 3,3’-diaminobenzidine (DAB) substrate with nickel (SK-4100; Vector Laboratories) following the manufacturer’s instructions. The reaction was stopped by rinsing the sections in PBS. Sections were mounted on chrome alum-gelatin coated slides, dehydrated through a graded series of ethanol, cover slipped, and viewed under brightfield microscopy.

Brain sections were analyzed using a Zeiss Axioplan 2 (4x and 10x magnification objectives) connected to a CX9000 high-resolution camera (MBF Bioscience, Williston, VT). Virtual slice images were obtained using the Stereo Investigator software (MBF Bioscience) and pixel densitometry was analyzed with ImageJ software (NIH, Bethesda, MD). Two regions of interest were used. The fimbria-hippocampal region of interest was applied to four serial sections from -0.94 mm to -3.16 mm bregma as defined using the mouse stereotaxic atlas by Paxinos and Franklin (2001). The septal region of interest was applied to serial sections from 1.10 mm to 0.38 mm bregma. This region was defined by a triangle that extended, dorso-ventrally, from the apex of the medial septum to a projected line connecting the lower edge of the anterior commissures on each hemisphere and, medio-laterally, from the midline to the outer limits of the medial septal area, as previously described (Lopez-Coviella et al., 2011). The intensity of each immunopositive cell profile within the region of interest was determined by measuring the mean gray intensity of the total area ($I_{\text{cell}}$) and the background profile ($I_{\text{bg}}$), determined by the average of 25 unique point measurements taken throughout the neuropil of each region of interest. The intensity profile of each region of interest was calculated using $1-(I_{\text{cell}}/I_{\text{bg}})*100$ and expressed as a percentage to be referred to as the immunoreactivity index (IR index) (Sia and Bourne, 2008).

2.3.6 Western Blot

Proteins (25 µg total protein per lane) from B6eGFPChAT, B6 and VACHT-KO hippocampal and caudate putamen brain homogenates were separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Trans-Blot
transfer medium; Bio-Rad Laboratories, Richmond, CA). The membranes were then blocked with tris buffered saline with tween-20 (TBST; 20 mM Tris HCl; 137 mM NaCl; 0.1% (v/v) Tween 20; pH 7.6) containing 5% skim milk. The membranes were washed in TBST and incubated with guinea pig anti-VACHT (AB1588, Millipore) overnight at 4°C. Following successive washing with TBST, the membranes were incubated with the appropriate horseradish peroxidase conjugated secondary antibody. Immunoreactive signals were detected using the SuperSignal West Dura enhanced chemiluminescence system (Pierce, Rockford, IL).

To quantify the relative amount of VACHT protein expression, blots were stripped and reprobed with antibodies against GAPDH (H86504M, Meridian Life Science, Memphis, TN) for 1 hour followed by a horseradish conjugated secondary antibody for an additional hour. Signal intensities were quantified using ImageJ software (NIH) and VACHT signal was normalized to GAPDH. The relative amount of VACHT protein in B6eGFPChAT tissue homogenates was expressed as a percent of VACHT protein present in B6 tissue.

2.3.7 Immunodot Assay

Proteins (1.8 µg total protein per dot) from B6eGFPChAT and B6 hippocampal, caudate putamen and cerebellar brain homogenates were loaded directly onto a nitrocellulose membrane (Trans-Blot transfer medium; Bio-Rad Laboratories). The membranes were air dried and blocked with TBST (20 mM Tris HCl; 137 mM NaCl; 0.1% (v/v) Tween 20; pH 7.6) containing 5% skim milk. The membranes were washed in TBST and incubated with guinea pig anti-VACHT (AB1588, Millipore) or GAPDH (H86504M, Meridian Life Science) overnight at 4°C. Following successive washing with TBST, the membranes were incubated with the appropriate horseradish peroxidase conjugated secondary antibody. Immunoreactive signals were detected using the SuperSignal West Dura enhanced chemiluminescence system (Pierce).

Signal intensities were quantified using ImageJ software (NIH) and VACHT signal was normalized to GAPDH. The relative amount of VACHT protein in B6eGFPChAT tissue homogenates was expressed as a percent of VACHT protein present in B6 tissue.
2.3.8 Choline Acetyltransferase Activity

B6eGFPChAT and B6 mice (13 weeks old, n=6 per group) were deeply anesthetised, sacrificed, and the brains were isolated and flash frozen in liquid nitrogen. The septal area was dissected on ice and sonicated in 5% (w/v) of homogenization buffer (HomB: 50 mM phosphate buffer; 10 mM ethylenediaminetetraacetic acid (EDTA); 1X protease inhibitor; 5% Triton X-100; pH 7.4). Samples were diluted so that assays were within linear range of the bovine serum albumin protein standard. [14C]Acetyl coenzyme A and choline were incubated with the tissue homogenate samples for 30 min at 37°C as per the method of Fonnum (1969) with slight modifications (Tucek et al., 1978) and as previously described (Cui et al., 2011). The reaction was stopped and the newly synthesized [14C]acetylcholine was extracted and counted in a liquid scintillation counter (Beckman Coulter, Mississauga, ON). Protein content of the samples was measured using the bicinchoninic acid (BCA) assay (Pierce).

2.3.9 Acetylcholinesterase Activity

B6eGFPChAT and B6 mice (13 weeks old, n=6 per group) were deeply anesthetised, sacrificed and the brains were quickly removed and hippocampi dissected. Acetylcholinesterase was extracted from the hippocampus by preparing a tissue homogenate in 5% trichloroacetic acid (TCA) through brief sonication and centrifugation to obtain a protein pellet. The TCA was removed with water-saturated diethyl ether, the sample was neutralized to pH 7.0 and the protein precipitate was reconstituted in 700 µL of HomB buffer. Acetylcholinesterase activity was determined using the Amplex Red detection kit (Invitrogen) by monitoring the conversion of Amplex Red to resorufin at 571 nm. Values were corrected to replicate samples treated with 0.1 mM physostigmine to adjust for endogenous choline and spontaneously hydrolyzing acetylcholine in the samples. Protein content of the samples was measured using the BCA assay (Pierce).

2.3.10 [14C]Acetylcholine Release in Hippocampal Slices

Hippocampal slices (500 µm) were prepared from freshly sacrificed B6eGFPChAT and B6 mice (13 weeks old, n=4 per group) and acetylcholine release was measured essentially as described (Barbosa et al., 1997) with slight modifications. Briefly, hippocampal slices were incubated in incubation buffer (124 mM NaCl; 4 mM KCl; 2 mM CaCl2; 25 mM HEPES; 10 mM Glucose;
1.2 mM MgSO₄) which was oxygenated with 100% O₂. Following incubation, slices were incubated in a depletion buffer (28 mM NaCl; 100 mM KCl; 2 mM CaCl₂; 25 mM HEPES; 10 mM Glucose; 1.2 mM MgSO₄) and then labelled in incubation buffer containing 0.01 mM physostigmine and 1µM [¹⁴C]choline. [¹⁴C]acetylcholine release was induced by incubating in depolarization buffer (62 mM NaCl; 66 mM KCl; 2 mM CaCl₂; 25 mM HEPES; 10 mM Glucose; 1.2 mM MgSO₄; 0.01 mM Physostigmine). The [¹⁴C]choline in the samples was oxidized to betaine using choline oxidase (Invitrogen) and the released [¹⁴C]acetylcholine was collected from the supernatant using sodium tetraphenylborate extraction (Cooper, 1989; Fonnum, 1969). Hippocampal slice pellets were digested with 5% TCA, and the radioactivity of both released and tissue samples were measured using liquid scintillation counting (Beckman Coulter). Protein content of the samples was measured using the BCA assay (Pierce).

2.3.11 Intracellular Acetylcholine

B6eGFPChAT and B6 mice (13 weeks old, n=4 per group) were deeply anesthetised, sacrificed, and the brains were quickly removed and hippocampi dissected. Acetylcholine and choline were extracted from the hippocampus by preparing a tissue homogenate in 5% TCA through brief sonication and centrifugation to obtain a protein pellet. The TCA was removed with water-saturated diethyl ether, vortex mixed and centrifuged for 10 min at 10,000 revolutions per minute (RPM). The aqueous phase was collected and neutralized to pH 7.0 with 6 N NaOH. Acetylcholine and choline contents were determined using the Amplex Red detection kit (Invitrogen). This assay detects acetylcholine through its specific degradation by AChE. Choline is then oxidized to betaine and H₂O₂ which can be quantified by monitoring the conversion of Amplex Red to resorufin at 571 nm. A serial dilution of acetylcholine standards and linear regression was used to model this relationship. Values were corrected to replicate samples treated with 0.1 mM physostigmine to maximally inhibit AChE and adjust for endogenous choline and spontaneously hydrolyzing acetylcholine in the samples. Protein content of the extracts was determined by reconstituting the protein pellet in HomB and assaying with the BCA assay (Pierce).
2.3.12 Statistical Analysis

GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) was used for statistical analysis and for the presentation of bar graphs. Independent two-tailed unpaired t-tests were used to compare the mean values of two groups. Two-way analysis of variance (ANOVA) was used to analyze data combining two independent variables. In all cases, significance was noted at \( *P < 0.05 \), \( **P < 0.01 \) and \( ***P < 0.001 \). Figure montages were created using Adobe Photoshop and Illustrator CS5 (Adobe Systems Inc., San Jose, CA).

2.4 Results

2.4.1 Replicated Cholinergic Gene Locus in the B6eGFPChAT Genome

In rodents, ChAT, VACHT and CHT are present as single copy genes per haploid genome on chromosomes 14 (ChAT and VACHT) and 17 (CHT). Using qPCR, we found that the genomic coding regions of both ChAT and VACHT are in 4-fold excess in the B6eGFPChAT genome compared to B6 controls (***\( P < 0.001 \), Fig. 2-1A,B) indicating that four copies of ChAT and VACHT are present per haploid B6eGFPChAT genome. No significant difference in the quantity of CHT genomic coding region was observed (Fig. 2-1C). These findings suggest that the transgene used to generate the B6eGFPChAT was inserted in triplicate following BAC engineering, as this sequence contained the entire coding regions for both ChAT and VACHT.

2.4.2 Elevated VACHT mRNA in the B6eGFPChAT Septum

To assess the effect of increased VACHT and ChAT gene copy number on the expression of cholinergic components, septal tissue from B6eGFPCHAT and B6 mice was used. qRT-PCR revealed a 4-fold elevation of VACHT mRNA expression in the septum of B6eGFPChAT mice compared to B6 (*\( P < 0.05 \), Fig. 2-2A). When ChAT expression was quantified, no significant change in expression was observed between genotypes (Fig. 2-2A). To confirm these results and examine other cholinergic markers, we performed semi-quantitative analysis. Using RT-PCR, we detected a single PCR product of expected size for each of our cDNA-specific primers targeting VACHT, ChAT, CHT and AChE (Fig. 2-2B). Quantification of band intensity showed that
Fig. 2-1. Quadruplication of the cholinergic locus in the B6eGFPChAT genome. A-C: Real-time amplification plots (top row) representing amplicons in B6eGFPChAT and B6 genomic DNA samples to quantify (bottom row) the DNA copy number per haploid genome for VACHT (A), ChAT (B) and CHT (C). VACHT and ChAT genes were found to be in 3- to 4-fold greater quantities in the B6eGFPChAT compared to B6 genome (***(P < 0.001). The CHT gene copy number was not statistically different in B6eGFPChAT compared to B6 genome. Values are means (range), based on REST convention, of gDNA isolated from four animals per group, each performed in triplicate.
VAChT mRNA levels were 2-fold greater in B6eGFPChAT compared to B6 mice (**P < 0.01, Fig. 2-2B). Quantification of the mRNA levels of ChAT, CHT and AChE revealed no significant differences in B6eGFPChAT compared to B6 mice (Fig. 2-2B). These experiments demonstrated that VAChT mRNA was uniquely overexpressed in the septum of B6eGFPChAT mice.

### 2.4.3 Enhanced VAChT Protein Content in the Hippocampus of B6eGFPChAT mice

To evaluate VAChT protein expression, we first compared VAChT immunostaining on coronal sections obtained from B6eGFPChAT and B6 mice. Qualitatively, stronger VAChT immunoreactivity was observed in all B6eGFPChAT mice compared to B6 mice in septohippocampal fibres in the medial septum (Fig. 2-3 A-C) as well as regions enriched in cholinergic projections, including the hippocampal formation, cortex, striatum and thalamic nucleus (Fig. 2-3 D). Quantification of the DAB signal intensity in the hippocampal formation indicates a 1.4-fold increase in VAChT immunoreactivity levels in B6eGFPChAT mice compared to B6 (**)P < 0.01, Fig. 2-3 E-H).

To further quantify the levels of VAChT protein in B6eGFPChAT mice, Western blotting was performed on brain homogenates. In both hippocampal and caudate putamen homogenates, VAChT protein was detected as a single diffuse band at the predicted size of 70 kDa (Fig. 2-4A,B). A secondary specific band was present at 35 kDa representing the proteolytic degradation product of VAChT (Gilmor et al., 1996). Both of these bands were absent in adult VAChT-KO homogenates, demonstrating their specificity. In hippocampal homogenates, quantification of the immunoreactivity for VAChT normalized to GAPDH revealed a 1.6-fold increase in B6eGFPChAT mice compared to B6 (*P < 0.05, Fig. 2-4A). A similar 1.5-fold increase was also observed in the caudate putamen of B6eGFPChAT mice compared to B6 (*P < 0.05, Fig. 2-4B). VAChT protein levels were also measured using an immunodot assay, which includes the immunoreactivity of both full-length VAChT and the 35 kDa degradation product of VAChT protein (Fig. 2-4C,D). This method also revealed a significant 1.7-fold increase in VAChT protein levels in the hippocampal formation (Fig. 2-4C) and caudate putamen (Fig. 2-4D) of
Fig. 2-2. Overexpression of VAChT mRNA and normal expression of ChAT, CHT and AChE mRNAs in B6eGFPChAT mice. A: qRT-PCR analysis demonstrating increased VAChT mRNA expression in the septum of B6eGFPChAT compared to B6 mice (*P < 0.05). No statistical differences were found in ChAT mRNA expression between genotypes. Values are mean percentages of B6 ± standard error of the mean (SEM) from three animals per group, each performed in triplicate. B: RT-PCR product levels were quantified using densitometry and normalized to GAPDH. The values are presented as mean percentages of B6 ± SEM of three animals per group, each performed in triplicate. Consistent with the data obtained by qRT-PCR, VAChT mRNA was overexpressed (**P < 0.01) with no statistical differences in ChAT mRNA in the septum of B6eGFPChAT compared to B6 mice. mRNA for CHT and AChE were also found to be not statistically different in septum of B6eGFPChAT compared to B6 mice. Below the graph are representative images of VAChT, ChAT, CHT and AChE RT-PCR results for B6 (left band) and B6eGFPChAT (right band) septal tissue. Amplicons were present as single bands at the expected sizes of 486 bp (VAChT), 520 bp (ChAT), 596 bp (CHT) and 435bp (AChE).
**Fig. 2-3.** Evaluation of VAChT expression in the hippocampus of B6eGFPChAT compared to B6 mice. A: Representative coronal section of the B6 (left) and B6eGFPChAT following VAChT immunoreactivity revealed by 3,3′-diaminobenzidine (VAChT-DAB). B and C: B6 (B) and B6eGFPChAT (C) septal images under 20x magnification illustrating increased VAChT immunoreactivity in B6eGFPChAT tissue. D: Representative coronal section of the B6 (left) and B6eGFPChAT (right) brain following VAChT-DAB. E: Schematic of the serial regions of interest for the quantification of hippocampal VAChT-DAB immunoreactivity. Anterior-posterior bregma coordinates are given below each section. F and G: Representative B6 (F) and B6eGFPChAT (G) fimbria-hippocampal region of interest selected for quantification. H: VAChT-DAB immunoreactivity index was significantly greater in hippocampus of B6eGFPChAT compared to B6 mice (**p < 0.01). The results are presented as a mean VAChT-DAB immunoreactivity index ± SEM of three animals using four sections per animal at the bregma levels indicated in E. MS, medial septum; LV, lateral ventricle; CPu, caudate putamen; th nu, thalamic nucleus; amyg nu, amygdala nucleus; cc, corpus callosum. Scale bar = 100µm.
B6eGFPChAT mice compared to B6 mice. Negative controls (cerebellar homogenates from B6 mice) revealed minimal VACHT immunoreactivity.

ChAT immunoreactivity indicated that ChAT protein levels were not statistically different in the brain of B6eGFPChAT mice compared to B6, as quantified in the septal area (Fig. 2-5). Consistent with the observed gene and protein expression, no significant differences in the levels of septal ChAT activity or hippocampal AChE activity were revealed in B6eGFPChAT compared to B6 mice (Fig. 2-6A,B).

Using three distinct approaches (immunohistochemistry pixel intensity, Western blot, and immunodot assay) we find a consistent ~1.5-fold increase in VACHT protein levels in B6eGFPChAT mice compared to B6 controls. No changes were found in ChAT protein or the levels of ChAT and AChE enzymatic activity between B6eGFPChAT and B6 mice indicating that the B6eGFPChAT mouse model is well suited for the specific study of VACHT overexpression in cholinergic neurons.

2.4.4 Enhanced Acetylcholine Release in the Hippocampus of B6eGFPChAT mice

We examined the influence of VACHT overexpression on basal and potassium-induced acetylcholine release in hippocampal slices collected from B6eGFPChAT and B6 mice. Potassium chloride depolarization significantly increased the release of [14C]acetylcholine from hippocampal slices in B6eGFPChAT and B6 preparations (F(1,12)=9.412, **P < 0.01, Fig. 2-7A). In addition, both the basal and potassium-induced acetylcholine release was elevated in B6eGFPChAT compared to B6 slices (F(1,12)=4.807, #P < 0.05, Fig. 2-7A). There was no significant interaction of potassium depolarization and genotype on acetylcholine release (F(1,12)=0.5259, P = 0.4822). Taken together, these data showed that acetylcholine release was enhanced in B6eGFPChAT mice. They further suggest that genotype does not affect the relative extent of acetylcholine released following potassium depolarization.

The levels of hippocampal intracellular acetylcholine were significantly lower in B6eGFPChAT compared to B6 mice (56% decrease compared to B6 values; *P < 0.05, Fig. 2-7B). This decrease in intracellular acetylcholine (Fig. 2-7B) was proportional to the increase in
Increased VACHT protein levels in the brain of B6eGFPChAT compared to B6 mice. A and B: Western blot analysis revealed significant increases in VACHT protein levels in hippocampal (A, 163% compared to B6; *P < 0.05) and caudate putamen (B, 153% compared to B6; *P < 0.05) homogenates of B6eGFPChAT compared to B6 mice (*P < 0.05). Immunoreactivity in VACHT-KO tissue was absent. The values are presented as mean percentages of B6 ± SEM of three animals per group. C and D: Immunodot analyses revealed a significant increase in VACHT protein levels in B6eGFPChAT hippocampal (C, 171% compared to B6; **P < 0.01) and caudate putamen (D, 172% compared to B6; **P < 0.01) brain homogenates. Cerebellum homogenates from B6 mice were used as a negative control due to their weak immunoreactivity, consistent with minimal VACHT protein in the cerebellum (Arvidsson et al., 1997). The values are presented as mean percentages of B6 ± SEM of three animals per group, each performed in triplicate.
Fig. 2-5. ChAT protein levels were not statistically different in the septum of B6eGFPChAT compared to B6 mice. A: Representative coronal section of the B6 (left) and B6eGFPChAT (right) brain following ChAT-DAB immunoreactivity. B: Schematic of the serial regions of interest for the quantification of septal ChAT-DAB immunoreactivity. Anterior-posterior bregma coordinates are given below each schematic section. C and D: Representative septal region of interest for quantification in B6 (C) and B6eGFPChAT (D) mice. E: Septal ChAT-DAB immunoreactivity index was not statistically different in B6eGFPChAT compared to B6 mice. The results are presented as a mean ChAT-DAB immunoreactivity index ± SEM of three animals using four sections per animal at the bregma levels indicated in B. Ctx, cortex; LV, lateral ventricle; CPu, caudate putamen; cc, corpus callosum; MS, medial septum. Scale bar = 250µm.
acetylcholine release in the hippocampus (Fig. 2-7A). In the context of our previous findings, this supports the mechanism that increased presynaptic VACHT elevates uptake of cytoplasmic acetylcholine, enhancing its release to the hippocampus in the B6eGFPChAT mouse.

2.5 Discussion

Here we present evidence to characterize the effect of VACHT overexpression on cholinergic function in the mammalian brain. In summary, B6eGFPChAT mice had increased VACHT gene copy number, resulting in elevated VACHT gene and protein expression in components of the septohippocampal pathway. VACHT overexpression led to enhanced acetylcholine release in the hippocampus, a region of the brain that receives cholinergic innervation from the septal area of the basal forebrain. In B6eGFPChAT mice, ChAT expression was normal. Therefore, we conclude that increased VACHT alone is sufficient to increase acetylcholine release.

Our first observation was the identification of multiple VACHT and ChAT gene copies in the B6eGFPChAT genome. The genomic information for VACHT and ChAT are localized to a common genomic locus collectively referred to as the cholinergic gene locus (for review, see Eiden, 1998). The cholinergic gene locus, including genomic regions upstream and downstream of this sequence, was the primary component of the BAC transgene that was engineered to generate the B6eGFPChAT mice. As such, we propose that the replication of VACHT and ChAT is due to multiple integration of the entire transgene itself. Indeed, previous reports have revealed that BAC transgenes typically integrate into the genome as concatemers, commonly up to five copies, that occur at a single random locus of the genome (Giraldo and Montoliu, 2001; Heaney and Bronson, 2006; Jaenisch, 1988; van Keuren et al., 2009). The precise process of BAC integration is unknown. A possible mechanism for gene replication may be attributed to BAC linearization occurring at multiple sites, homologous recombination of the multiple BAC fragments into concatemers and homologous illegitimate recombination of the fused product into the target genome. Regardless of the mechanism, these initial findings highlight important considerations in using the B6eGFPChAT mice for the study of normal cholinergic function, as B6eGFPChAT mice may have been expected to have normal levels of VACHT expression.
Fig. 2-6. B6eGFPChAT compared to B6 mice. A and B: Septal ChAT (A) and hippocampal AChE (B) enzymatic activity in B6 and B6eGFPChAT mice. Values are the means ± SEM of six animals per group, each performed in duplicate.

Fig. 2-7. Enhanced acetylcholine release in the hippocampus of B6eGFPChAT mice. A: Basal and potassium-induced acetylcholine release was significantly elevated in hippocampal slices from B6eGFPChAT mice compared to B6 controls (#P < 0.05). Potassium depolarization increased acetylcholine release in hippocampal slices from both B6eGFPChAT and B6 mice (***P < 0.01). Values are mean acetylcholine released as a percentage of total radioactivity ± SEM of four animals, each performed in duplicate. B: Intracellular acetylcholine content was significantly reduced in the hippocampus of B6eGFPChAT compared to B6 mice (*P < 0.05). Values are mean intracellular acetylcholine content ± SEM of four animals, each performed in triplicate.
Multiple gene copies have been strongly associated with enhanced transgene expression for numerous independent transgenic mice derived from several distinct BAC transgenes (Chandler et al., 2007). In this study, we observed increased VACHT mRNA expression in the septal area, a native site for robust cholinergic expression and the site of gene transcription for cholinergic neurons of the septohippocampal pathway. We considered the possibility that ChAT could be overexpressed to a similar extent as VACHT. However, by design of the BAC transgene, ectopic ChAT expression is terminated by the inclusion of a poly(A) signal sequence downstream from the eGFP coding region (Kim and Martinson, 2003; Tallini et al., 2006). This is consistent with our findings that ChAT mRNA expression was unaffected in the B6eGFPChAT mice. To address the possibility that the poly(A) sequence was not completely effective in terminating transcription, we performed a western blot using antibodies against the eGFP protein that revealed a single band at ~30 kDa as expected for non-fused eGFP protein (data not shown). Under certain circumstances, it is clear that VACHT and ChAT transcripts may be co-regulated (Berse and Blusztajn, 1995; Eiden, 1998; Schütz et al., 2001). However, the complex regulation of the cholinergic gene locus also allows for ChAT- and VACHT-specific mRNA production from unique promoters or through the activity of alternative RNA splicing (Misawa et al., 1997; Oda, 1999), providing support for the isolated VACHT overexpression observed here.

We were interested to see whether, aside from ChAT, increasing VACHT expression affected the transcriptional profile of other presynaptic cholinergic markers. Previous studies have characterized the complex regulatory nature of cholinergic neurons revealing compensatory transcriptional changes following the manipulation of ChAT (Brandon et al., 2004; Matsuo et al., 2011), CHT (Ferguson et al., 2004) and AChE (Breer and Knipper, 1990; Hartmann et al., 2008; Volpicelli-Daley et al., 2003). In the present study, we show that VACHT overexpression has no apparent effect on the expression level of CHT or AChE. These findings are in agreement with observations by Prado and colleagues who showed that VACHT-deficient mice present no significant difference in CHT expression, choline transport or markers of acetylcholine production (Prado et al., 2006).

In addition to increased VACHT gene expression, we detected increased VACHT protein levels in several cholinergic regions of the brain including the hippocampus, a primary axonal target for septal cholinergic neurons. This suggests that in B6eGFPChAT mice, the hippocampal protein
levels of VACHT closely follows that of septal VACHT mRNA, in agreement with the established association between cholinergic gene transcription and translation (Holler et al., 1996). To evaluate the effect of increased presynaptic VACHT on cholinergic neurotransmission, we measured the spontaneous and potassium-induced release of acetylcholine from cholinergic terminals in the hippocampus. Potassium depolarization was chosen because it induces an indiscriminate depletion of acetylcholine from the synaptic boutons and provides an assessment of the complete acetylcholine stores, including those that occupy the reserve pool of presynaptic vesicles (Rizzoli and Betz, 2005; Takei et al., 1996). We found increases in both the spontaneous and potassium-induced acetylcholine release in B6eGFPChAT mice. We reasoned that increased acetylcholine release could either be due to increased acetylcholine production, or increased packaging of existing acetylcholine. However, consistent with the observed levels of transcription, ChAT protein levels and the enzymatic activity of both ChAT and AChE were unchanged in B6eGFPChAT tissue. These findings indicate that changes in acetylcholine synthesis are unlikely to contribute to the increased hippocampal acetylcholine release exhibited by B6eGFPChAT mice. We therefore proposed that the increased acetylcholine release was the result of enhanced VACHT-mediated acetylcholine packaging into synaptic vesicles.

In hippocampal homogenates, we observed decreased intracellular acetylcholine content in B6eGFPChAT mice compared to B6 controls. We propose that the decreased intracellular acetylcholine levels observed here can be explained by the enhanced packaging of cytosolic acetylcholine and vesicle-mediated efflux of acetylcholine to the synaptic cleft in B6eGFPChAT mice. This interpretation is consistent with findings related to the increased volume of acetylcholine-containing synaptic vesicles in VACHT-overexpressing Xenopus embryos (Song et al., 1997). Furthermore, our findings on the impact of VACHT overexpression are, as expected, opposite to those observed in heterogeneous and homogenous VACHT-deficient mice. VACHT-deficient transgenic mice exhibit decreased basal and potassium evoked acetylcholine release in the striatum due to insufficient filling of synaptic vesicles (Prado et al., 2006). In these models, the decreased synaptic packaging of acetylcholine led to enhanced intracellular acetylcholine in the cytosol (Prado et al., 2006). When considering these studies along with our findings, it is evident that both VACHT overexpression and deficiency have a significant impact on the regulation of cholinergic tone.
It remains to be assessed whether the VACHT-mediated enhancement of acetylcholine packaging can be sustained by sufficient acetylcholine synthesis. Earlier studies have demonstrated in rodents and humans that a reduction of ChAT expression by up to 50% is well tolerated (Brandon et al., 2004; Ohno et al., 2001; Wenk et al., 1986). This highlights the view that under normal conditions, ChAT is present in kinetic excess in cholinergic neurons. During VACHT overexpression in B6eGFPChAT mice, normal activity and expression levels of ChAT and CHT were maintained. This suggests that during VACHT overexpression, acetylcholine synthesis may have the capacity to sustain enhanced acetylcholine release by capitalizing on the inherent properties of cholinergic neurons to synthesize excess acetylcholine. Future electrophysiological and behavioural studies will determine the effects of VACHT overexpression on cholinergic neurotransmission and related functions.

With regards to the potential of VACHT as a therapeutic target for cholinergic dysfunction, it is of interest to note that declining levels of VACHT have been reported during AD progression (Bell and Cuello, 2006) with significant reductions in VACHT mRNA and protein in post-mortem AD brains (Chen et al., 2011a; Efange et al., 1997). At present, AChE inhibitors are used as therapeutic intervention in AD to prevent the breakdown of acetylcholine at the synaptic cleft and prolong cholinergic receptor activation. However, the use of AChE inhibitors does not always result in cognitive improvement (Lanctôt et al., 2003). It is possible that the amount of acetylcholine produced and released has become insufficient to sustain optimal cholinergic function, even in the presence of AChE inhibition. Our data suggest that acetylcholine release can be enhanced by increasing VACHT expression, which may be necessary to reach adequate levels of cholinergic neurotransmission. As AD is characterized by deficits in several neurotransmitter systems (Lanari et al., 2006), a more global approach to increase the release of several neurotransmitters in the brain may also be necessary. Similar to our findings that result from VACHT overexpression, enhancing the expression of other transporters including the vesicular glutamate transporter, excitatory amino acid transporter, the serotonin reuptake transporter, and the dopamine reuptake transporter may be sufficient to improve the deficits observed in other neurotransmitters systems (Chen et al., 2011a).

In summary, our findings support the role of VACHT in regulating acetylcholine release and provide novel evidence that the overexpression of VACHT in septal cholinergic neurons results
in enhanced acetylcholine release in the hippocampus. Normal levels of expression and activity were maintained for other components of the cholinergic system (i.e. ChAT, CHT and AChE). Enhancing VACht expression alone is therefore sufficient to increase acetylcholine release. These findings can be of significance in the design of therapeutic approaches to increase the neurotransmission of cholinergic neurons and perhaps other neuronal phenotypes utilizing additional vesicular transport systems.
Chapter 3
B6eGFPChAT mice overexpressing the vesicular acetylcholine transporter exhibit spontaneous hypoactivity and enhanced exploration in novel environments

Contents of this chapter have been published:


P.M.N. led and conducted all experiments and drafted the manuscript. I.A. provided materials, analysis tools and experimental design as well as manuscript revision.
3.1 Abstract

Cholinergic innervation is extensive throughout the CNS and PNS. Among its many roles, the neurotransmitter acetylcholine contributes to the regulation of motor function, locomotion and exploration. Cholinergic deficits and replacement strategies have been investigated in neurodegenerative disorders, particularly in cases of AD. Focus has been on blocking AChE and enhancing acetylcholine synthesis to improve cholinergic neurotransmission. As a first step in evaluating the physiological effects of enhanced cholinergic function through the upregulation of VACt, we used the hypercholinergic B6eGFPChAT congenic mouse model that has been shown to contain multiple VACt gene copies. Analysis of biochemical and behavioural paradigms suggest that modest increases in VACt expression can have a significant effect on spontaneous locomotion, reaction to novel stimuli and the adaptation to novel environments. These observations support the potential of VACt as a therapeutic target to enhance cholinergic tone, thereby decreasing spontaneous hyperactivity and increasing exploration in novel environments.

3.2 Introduction

Cholinergic neurotransmission plays key roles in the CNS and PNS (Woolf and Butcher, 2011). Cholinergic impairments in neurodegenerative diseases, especially in AD, have led to the development of several cholinergic-based therapeutic strategies. Aside from the well-characterized role of acetylcholine in cognitive functions such as learning and memory, acetylcholine availability has been shown to contribute to a number of physiological and behavioural functions including peripheral motor function (Ribeiro et al., 2006; Woolf and Butcher, 2011) and locomotor activity (Di Chiara et al., 1994; Martins-Silva et al., 2011; Woolf and Butcher, 2011). Specifically, clinical assessments and experimental models of AD revealed that decreased cholinergic tone can cause spontaneous hyperactivity including increased restlessness, coupled with increased anxiety in novel environments (Bedrosian et al., 2011; McGuinness et al., 2010; Ognibene et al., 2005; Piccininni et al., 2005; Sterniczuk et al., 2010b; Walker et al., 2011). Therefore, strategies to modify cholinergic tone may provide a means to regulate both spontaneous and novelty-induced locomotion (Mega et al., 1999).
Cholinergic neurotransmission is maintained through the appropriate synthesis, vesicular packaging and release of acetylcholine. Choline, sequestered through CHT, is transacetylated via the enzymatic activity of ChAT and the precursor acetyl-coenzyme A (reviewed in Blusztajn and Wurtman, 1983). Newly synthesized acetylcholine is packaged into synaptic vesicles by VACHT prior to its release to the synaptic cleft (Parsons, 2000).

Genetic targeting has been used to create mouse models presenting deficiency in one or more cholinergic components, including VACHT (de Castro et al., 2009a; Guzman et al., 2011; Martins-Silva et al., 2011; Prado et al., 2006), ChAT (Brandon et al., 2004; Misgeld et al., 2002), CHT (Bazalakova et al., 2007), AChE (Volpicelli-Daley et al., 2003) or through the modified expression of acetylcholine receptors (Drenan et al., 2008; Drenan et al., 2010; Picciotto et al., 2000; Wess et al., 2007). Until recently, most animal models of cholinergic enhancement have been limited to the pharmacological inhibition of acetylcholine degradation in the synaptic cleft. The previously characterized B6eGFPChAT mouse model (Nagy and Aubert, 2012; Tallini et al., 2006) allows for the evaluation of whether increasing the vesicular storage and release of acetylcholine is sufficient to elicit changes in behavioural activity. B6eGFPChAT mice have four genomic copies of the cholinergic gene locus, which contains the VACHT and ChAT promoter and coding regions (Eiden, 1998; Nagy and Aubert, 2012; Tallini et al., 2006). In these mice, the transcription of transgenic ChAT is terminated and replaced by the eGFP, while the transcription of the VACHT transgene remains operational. As such, VACHT is overexpressed, while levels of ChAT, CHT and AChE are maintained, in cholinergic neurons (Nagy and Aubert, 2012).

Here, the behaviour of B6eGFPChAT mice was assessed in a panel of tests designed to elicit a variety of central and peripheral responses. We found that B6eGFPChAT mice have enhanced spontaneous activity and novelty induced exploration. The results of this study support the notion that modulating VACHT levels modifies behavioural activity, highlighting the importance of acetylcholine vesicular storage in the regulation of cholinergic neurotransmission and function.

3.3 Materials and Methods

3.3.1 Animals

For all studies, congenic male B6.Cg-Tg(RP23-268L19-EGFP)2Mik/J (B6eGFPChAT; Jackson Laboratories, Bar Harbour, ME, USA) mice homozygous for the RP23-268L19-EGFP transgene
were compared with sex and age-matched B6 controls. Separate cohorts of animals were used for the biochemical, immunohistological and behavioural studies. For the behavioural panel, B6eGFPChAT (N=11) and B6 (N=9) mice were between 124 – 126 days of age at upon entry to this study, housed under identical conditions, and exposed to regular handling prior to and during the study. The behavioural panel was conducted sequentially in the following order: OF (Day 1-5), peripheral motor function (Day 9), Rotorod (Day 10-11), dark/light box (Day 18) and EPM (Day 48). A subset of this cohort (N=8 per genotype) were used for calorimetry (Day 24-28; Day 37-41). Presence of the transgene was confirmed using conventional PCR and primers as previously described (Tallini et al., 2006), and by the expression of eGFP observed during immunofluorescence microscopy protocols. All animal protocols were approved by the Animal Care Committees of Sunnybrook Research Institute and the University of Western Ontario, and experiments were performed according to the guidelines set by the Canadian Council on Animal Care and the Animals for Research Act of Ontario.

3.3.2 Immunofluorescence microscopy

B6eGFPChAT mice were concurrently anaesthetized with a mixture of ketamine (75 mg/kg) and xylazine (10 mg/kg). The mice were then perfused intracardially with saline, followed by fixation with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed, post-fixed overnight and equilibrated in 30% sucrose. Coronal sections were cut at 30 µm and collected in 96-well plates filled with cryoprotectant solution (50 mM phosphate buffer; 25% (v/v) glycerin; 30% (v/v) ethylene glycol; pH 7.4). Sections were blocked using 0.3% bovine serum albumin in phosphate buffered saline and incubated with a primary antibody against VAChT (guinea pig polyclonal AB1588; 1:1000 dilution; Millipore, Temecula, CA, USA) followed with a donkey anti-guinea pig Cy3 antibody to reveal VAChT immunoreactivity (1:500; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Fluorescent labelling was detected by confocal microscopy (Zeiss Axiovert 100M, LSM 510; Carl Zeiss, Don Mills, Canada).

3.3.3 Western blot

Proteins (25 µg total protein per lane) from B6eGFPChAT (N=3) and B6 cortical (N=3), striatal and hippocampal brain homogenates were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose (Trans-Blot transfer medium; Bio-Rad Laboratories, Richmond, CA, USA) or polyvinylidene fluoride membrane
(Immobilon-P, Millipore). The membranes were then blocked with TBST (20 mM Tris HCl; 137 mM NaCl; 0.1% (v/v) Tween 20; pH 7.6) containing 5% skim milk. The membranes were washed in TBST and incubated with guinea-pig anti-VACHT (AB1588, Millipore), anti-ChAT (AP144P, Millipore) or anti-CHT (AB5966, Millipore) antibodies overnight at 4°C. Following successive washing with TBST, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. Immunoreactive signals were detected using the SuperSignal West Dura enhanced chemiluminescence system (Pierce, Rockford, IL, USA).

To quantify the relative amount of protein expression, blots were stripped and reprobed with antibodies against GAPDH (H86504M, Meridian Life Science, Memphis, TN, USA) for 1 h followed by a horseradish peroxidase-conjugated secondary antibody for an additional hour. Signal intensities were analyzed using GeneTools software (Syngene, Frederick, MD, USA) and normalized to GAPDH. The relative amount of VACHT, ChAT and CHT protein in B6eGFPChAT tissue homogenates was expressed as a percent of protein present in B6 control tissue. Mean normalized densitometry values were analyzed by Student’s t-test to compare genotypes.

3.3.4 Spontaneous activity and indirect calorimetry

B6eGFPChAT (N=8) and B6 (N=8) mice were placed in comprehensive lab animal monitoring system metabolic cages (Columbus Instruments, Columbus, OH, USA). These metabolic chambers monitor activity and metabolic performance. Following entry into the cages, the mice were allowed to acclimatize to the environment for 14-17 h prior to data collection. High-resolution real time activity data along with metabolic measurements collected every 10 minutes were acquired during the 12 h light cycle (07:00 and 19:00 h) and 12 h dark cycle (19:00 and 07:00 h). The metabolic measurements included the VCO₂, VO₂, RER and the caloric (heat) value ([(3.815 + 1.232 x RER) x VO₂] x 1000)/mouse weight). Sleep analysis was conducted using the Oxymax software (Columbus Instruments, Columbus, OH, USA) as previously described and validated (Pack et al., 2007). The sleep threshold was set to 180 seconds of ≤10 activity counts. The data are represented in approximately 30 minute intervals and analyzed using repeated measures two-way ANOVA or as the mean values over each 12 h period and analyzed using Student’s t-test.
3.3.5 Dark/Light Box

Each mouse was placed into an automated activity monitor (Accuscan Instruments, Inc., Columbus, OH, USA) that was separated into an enclosed dark region (21 x 42 cm) and an open light region (21 x 42 cm). The two regions were separated by an opening (10 x 15 cm) where mice were placed facing the dark region and allowed to explore for 10 minutes between 20:00 and 22:00 h. Activity (converted from infrared beam breaks to cm) in each of the two regions along with transitions between the regions were measured over the trial duration. Mean distance values were analyzed by Student’s \( t \)-test. The proportional time and distance spent in the light field and the number of transitions were analyzed by the Mann-Whitney U test.

3.3.6 Novel Environment Locomotion

Locomotor activity was measured using an automated activity monitor (Accuscan Instruments, Inc., Columbus, OH, USA). Experiments were performed between 10:00 and 16:00 h. Mice were allowed to explore the locomotor activity chamber (42 x 42 x 30 cm) for 2 h. Activity (converted from infrared beam breaks to cm) was measured at 5-min intervals. Measurements of activity were analyzed using repeated measures two-way ANOVA while cumulative means were assessed by the Student’s \( t \)-test.

3.3.7 Elevated Plus Maze

Anxiety-like and exploratory behaviour was evaluated using an EPM 72 cm above the floor with four arms 50.8 cm long and 10.2 cm wide (two darkened and enclosed with 40.6 cm walls). Mice were placed into the center of the maze facing one of the open arms. The accumulated time and distance spent on the open and closed arms, along with the entries into each of the arms was recorded over a single trial of 5 minutes using the automated tracking system (AnyMaze, Stoelting, Wood Dale, IL, USA). The percentage of time spent on each of the arms and the percentage of entries into the arms were analyzed using Student’s \( t \)-test or Mann-Whitney U test as parameters measuring anxiety-like behaviour.

3.3.8 Rotarod

Mice were placed on a stationary rod of an automated rotarod apparatus (SD Instruments, San Diego, CA, USA). The rotation of the rod was then initiated at the speed of 5 RPM, which accelerated at a rate of 10 RPM/minute to 35 RPM over the course of 3 minutes. Latency to fall
was automatically recorded using infrared beam break as the animal fell from the rod. Mice were tested on ten trials during the first day, and four trials the next day, each with 15-minute inter-trial intervals. Results were analyzed by repeated measures ANOVA.

3.3.9 Grip Strength

Forelimb grip strength was measured using a horizontally mounted digital force gauge (Chatillon, Largo, FL, USA). Mice held by the base of their tails were slowly lowered and allowed to grasp a triangular bar attached to the gauge. The mice were then pulled backwards along the horizontal plane of the gauge. The peak tension of ten successive trials was collected. Mean peak tension results for each genotype were analyzed by Student’s t-test.

3.3.10 Hanging Wire

Each mouse was placed on a wire cage top (square ½ inch mesh) which was gentle shaken once to encourage the mice to grasp. The wire cage top was slowly inverted and suspended 40 cm above the base of a padded Plexiglas box. The mice were given three trials up to 300 seconds with an inter-session interval of 30 seconds. The time it took each mouse to fall from the cage top was recorded. The mean trial hanging time results for each genotype were analyzed using repeated measures ANOVA and mean cumulative hang time over each of the trials were analyzed by Student’s t-test.

3.4 Results

3.4.1 VACHT is overexpressed in the B6eGFPChAT mouse

In the cortex, striatum and hippocampus, VACHT staining presented as punctate fluorescence along ChAT positive fibers and in cell bodies of the striatum (Fig. 3-1A). Our previous observations in 3 month-old B6eGFPChAT mice (Nagy and Aubert, 2012) revealed enhanced VACHT protein expression and here, we confirm that at 6 months of age, VACHT overexpression is sustained. The expression of VACHT in B6eGFPChAT mice was compared to B6 controls using Western blot analysis to detect cholinergic immunoreactivity in various regions of the CNS. Western blot targeting VACHT revealed a diffuse doublet at the predicted size of 70 kDa (Fig. 3-1B). Quantification of the VACHT band intensity revealed a significant 2- to 3-fold increase of VACHT protein in B6eGFPChAT compared to B6 control mice (Fig. 3-1C). The enhanced level of VACHT protein was found in the cortex ($t(4) = 8.752; P = 0.001$), striatum ($t(4)$)}
= 4.494; P = 0.046) and hippocampal formation (t(4) = 5.323; P = 0.006) (Fig. 3-1D). Western
blots and quantification of ChAT (Fig. 3-1D,E) and CHT (Fig. 3-1F,G) revealed no significant
change in protein expression in any of the regions that were analyzed.

3.4.2 B6eGFPChAT mice exhibit unaltered motor function and coordination

To measure the effect of increased VACHT on peripheral motor function, we first assessed
forelimb grip strength using a digital tension gauge. B6eGFPChAT mice produced a peak
tension of 0.268 kg which was not found to be significantly different from B6 control mice that
produced 0.260 kg of peak tension (t(18) = 0.416; P = 0.682) (Fig. 3-2A). In addition, no
statistical difference was found between B6eGFPChAT and B6 control mice when measuring
wire hang fatigue (two-way repeated measures ANOVA revealed no significant genotype factor,
F(1,36) = 0.052; P = 0.822 and the expected trial factor, F(2,36) = 11.04; P < 0.001) (Fig. 3-2B) or
total hanging time performance (t(18) = 0.229; P = 0.822) (Fig. 3-2C).

We considered that the effect of VACHT overexpression might only be detectable during
activities combining endurance, fine motor coordination and balance. As such, performance on
the rotarod was assessed through the latency to fall off the rotating cylinder. Both B6eGFPChAT
and B6 control mice improved significantly from trial 1 to trial 10 (two-way repeated measures
ANOVA trial factor, F(9,162) = 8.653; P < 0.001), demonstrating that both strains significantly
improved motor coordination over time (Fig. 3-2D). However, no significant effect of genotype
(F(1,162) = 0.013; P = 0.910) or interaction (F(9,162) = 1.273; P = 0.256) were found. Motor skill
retention was assessed using four additional trials 24 hours following the training sessions. In
this paradigm, no significant differences were found between the latency to falling during Trial
10 (Day 1) and Trial 11 (Day 2) for B6eGFPChAT or B6 control mice (two way repeated
measures ANOVA F(1,18) = 0.201; P = 0.659). Similarly, no genotype effect on performance was
observed during the four trials performed during Day 2 (F(1,54) = 0.366; P = 0.553) (Fig. 3-2D).
Taken together, these data suggest that B6eGFPChAT mice have maintained motor function and
learning compared to B6 control mice, and that elevated VACHT mediated acetylcholine
vesicular packaging as observed in B6eGFPChAT mice is not sufficient to improve these normal
motor functions.
Fig. 3-1. VACht protein is overexpressed in B6eGFPChAT mice. A: RP23-268L19-EGFP transgene is expressed throughout the central nervous system of B6eGFPChAT mice including cortical, striatal and hippocampal regions (green). Punctate VACht immunoreactivity is present in cholinergic cell bodies and processes (red). B and C: Representative immunoblots for VACht (B) and densitometry quantification (C) of immunoblot reveals significant 2- to 3-fold overexpression of VACht in the cortex, striatum and hippocampus of B6eGFPChAT mice compared to B6 control mice. D and E: Representative immunoblot for ChAT (D) and densitometry quantification (E) shows no significant difference in ChAT expression between genotypes in the analyzed regions. F and G: Representative immunoblot for CHT (F) and densitometry quantification (G) shows no significant difference in CHT expression between genotypes in the analyzed regions. * p<0.05; ** p<0.01; *** p<0.005 compared to B6 control mice.
3.4.3 B6eGFPChAT mice display spontaneous hypoactivity in a home cage environment

Given the role of cholinergic neurons in the regulation of muscle activity through central and peripheral innervation, we sought to determine whether increased VAChT expression influences spontaneous locomotor activity. Through the monitoring of locomotor activity over a 24 hour period, B6eGFPChAT mice were found to exhibit hypoactivity during both their light (t\(_{(14)}\) = 2.205; P = 0.045) and dark cycles (t\(_{(14)}\) = 3.823; P = 0.002) (Fig. 3-3A). High-resolution analysis of the locomotor activity exposed a significant genotype factor when analyzed by repeated measures two-way ANOVA (F\(_{(1,658)}\) = 4.660; P = 0.049) (Fig. 3-3B). Bonferroni post-test revealed that the B6eGFPChAT mice displayed significantly less activity during the biphasic diurnal activity peaks typically exhibited by rodents at approximately 21h00 and 4h30 (Fig. 3-3B). We further evaluated physiological function and tone through the assessment of respiratory characteristics that are associated with physical activity. Using two-way repeated measures ANOVA, we found that there was no significant genotype effect during the assessment of RER (F\(_{(1,658)}\) = 2.105; P = 0.169) (Fig. 3-3C), Heat (F\(_{(1,658)}\) = 0.502; P = 0.491) (Fig. 3-3D), VO\(_2\) (F\(_{(1,658)}\) = 0.418; P = 0.528) (Fig. 3-3E) and VCO\(_2\) (F\(_{(1,658)}\) = 0.038; P = 0.848) (Fig. 3-3F). Considering the time points where statistically significant decreases in activity occurred (between 20h30 – 23h00), a corresponding statistically significant decrease in VO\(_2\) in B6eGFPChAT mice (F\(_{(1,70)}\) = 5.784; P = 0.031) (Fig. 3-3E) was observed. These results show that B6eGFPChAT mice are spontaneously hypoactive in familiar environments and that locomotion under these conditions is dependent on the level of expression of the vesicular acetylcholine transport.

3.4.4 Normal distribution of sleep and wakefulness in B6eGFPChAT mice

Sleep analysis performed using B6eGFPChAT and B6 control mice home cage activity data did not identify significant genotype factors for the percentage of sleep time (F\(_{(1,28)}\) = 0.005; P = 0.942) (Fig. 3-4A), average sleep bout duration (F\(_{(1,28)}\) = 0.389; P = 0.538) (Fig. 3-4B) and the total number of sleeping bouts (F\(_{(1,28)}\) = 0.771; P = 0.387) (Fig. 3-4C). In addition, the nocturnal preference for wakefulness was maintained between genotypes given the significant cycle factor for sleep time (F\(_{(1,28)}\) = 363.7; P < 0.001), average sleep bout duration (F\(_{(1,28)}\) = 16.87; P < 0.001)
Fig. 3-2. Neuromuscular function and coordination in B6eGFPChAT mice. A: Maximum forelimb grip strength measured in B6eGFPChAT (N=11) and B6 control mice (N=9). B and C: Time spent hanging upside-down from a wire grid in each of three consecutive trials (B) and cumulative time (C) for B6eGFPChAT (N=11) and B6 control mice (N=9). D: Latency to fall from an accelerating rod (5 – 35 RPM; rate of 10 RPM/min) over 10 trials on day 1, followed by 4 trials on day 2. ### p<0.005 compared to trial 1.
Fig. 3-3. Spontaneous activity and indirect calorimetry of B6eGFPChAT mice. A: Cumulative horizontal beam break activity over the total each of the 12 hour light and dark cycles for B6eGFPChAT (N=8) and B6 control mice (N=8). B: Home cage horizontal beam break activity recorded in five minute intervals over twenty-four hours for B6eGFPChAT (N=8) and B6 control mice (N=8). C: Respiratory exchange ratio recorded in thirty minute intervals over twenty-four hours for B6eGFPChAT (N=8) and B6 control mice (N=8). D: Heat generation recorded in thirty minute intervals over twenty-four hours for B6eGFPChAT (N=8) and B6 control mice (N=8). E: VO₂ consumed in thirty minute intervals over twenty-four hours for B6eGFPChAT (N=8) and B6 control mice (N=8). F: VCO₂ generated in thirty minute intervals over twenty-four hours for B6eGFPChAT (N=8) and B6 control mice (N=8). Light-dark cycle conditions are distinguished using monochromatic background fill. * p<0.05; *** p<0.005 compared to B6 controls.
and total number of sleep bouts ($F_{(1,28)} = 24.90; P < 0.001$). No significant interaction factors were observed for sleep time, duration or bouts. These data suggest that the duration and circadian patterns of sleep are unaltered by VACHt overexpression in B6eGFPChAT compared to B6 mice.

### 3.4.5 B6eGFPChAT mice display increased activity and exhibit impaired habituation in novel environments

To evaluate the behavioural response to a novel environment, we placed B6eGFPChAT and B6 control mice into OF arenas for two hours. To establish the instantaneous response to novelty, we first considered the data collected during the initial five minutes of exposure which has been previously established as a predictive time to establish the effect (Crawley, 2007). Using this criteria, B6eGFPChAT mice exhibit a significant increase in total distance ($t_{(18)} = 3.199; P = 0.005$) (Fig. 3-5A) and rearing activity ($t_{(18)} = 2.570; P = 0.019$) (Fig. 3-5C) compared to B6 controls.

Activity plots over the 2 hour duration of the initial exposure to the novel environment do not reveal significant differences in the intra-session habituation between B6eGFPChAT and B6 controls as expressed by total distance (two-way repeated measures ANOVA revealed significant effect of time, $F_{(23,414)} = 40.40; P < 0.001$, but no effect of genotype, $F_{(1,414)} = 1.210; P = 0.286$, or interaction, $F_{(23,414)} = 1.495; P = 0.067$) (Fig. 3-5A). Consistently, habituation expressed by rearing events was not statistically different between B6eGFPChAT and B6 control mice (no effect of genotype, $F_{(1,414)} = 0.445; P = 0.513$, or interaction, $F_{(23,414)} = 1.302; P = 0.160$ and the expected significant effect of time in both strains, $F_{(23,414)} = 31.53; P < 0.001$) (Fig. 3-5C).

In addition to a single exposure, we tested inter-session habituation by repeating the exposure of mice to the boxes in three consecutive days (Fig. 3-5B,D). B6 mice exhibited a decrease in total activity which reached statistical significance during day 3 when compared to day 1 ($F_{(2,26)} = 5.232; P = 0.013$) (Fig. 3-5B; light bars). In contrast, B6eGFPChAT mice did not show statistically significant changes in total distance between exposures (Fig. 3-5B; dark bars). Notably, B6eGFPChAT mice revealed significantly higher locomotion when compared to B6 control mice during the day 3 exposure (Bonferroni post-hoc test between B6eGFPChAT and B6 control on day 3, $t = 2.884; P = 0.013$) (Fig. 3-5B). No significant difference was observed for
Fig. 3-4. Temporal sleep patterns in B6eGFPChAT mice. A: Proportion of time spent sleeping during each of the light, dark and combined cycles over a twenty-four hour period in B6eGFPChAT (N=8) and B6 control mice (N=8). B: Sleep bout duration during each of the light, dark and combined cycles over a 24 hour period in B6eGFPChAT (N=8) and B6 control mice (N=8). C: Total number of sleep bouts during each of the light, dark and combined cycles over a 24 hour period in B6eGFPChAT (N=8) and B6 control mice (N=8).
habituation of rearing events (no genotype effect, $F_{(1,36)} = 1.405; P = 0.251$, expected time effect, $F_{(2,36)} = 17.25; P < 0.001$) (Fig. 3-5D). From these data, we show that B6eGFPChAT mice exhibit increased locomotor activity upon initial exposure to OF environments, which decreases to B6 levels by 10 minutes and is followed by maintained intra-session habituation. In addition, B6eGFPChAT mice were found to have increased locomotor activity compared to B6 controls during the day 3 exposures.

3.4.6 Thigmotactic behaviour is maintained in B6eGFPChAT mice

We considered that the brief increase in locomotor behaviour exhibited in the OF environment might be due to differences in anxiety in B6eGFPChAT compared to B6 mice. We therefore sought to evaluate the thigmotactic behaviour of the B6eGFPChAT mice (i.e. the proportion of time spent along the periphery of the OF) during a novel exposure to the environment. No significant difference was observed during the first 5 minutes ($t_{(18)} = 0.3479; P = 0.732$), or during the 2 hour duration with regards to the proportion of time spent in the center between the B6eGFPChAT and B6 genotypes (two-way repeated measure ANOVA did not reveal a significant genotype factor, $F_{(1,414)} = 0.5771; P = 0.457$) (Fig. 3-6A). We did observe, however, a significant interaction in the proportion of center time between B6eGFPChAT and B6 control mice ($F_{(1,414)} = 4.000; P < 0.001$). Through visual inspection of the data in Fig. 3-6A, we hypothesized that the interaction effect was due to increased unbiased activity during the last hour of the trial. As such, we generated activity maps for the first and second hours of the exposure to compare the activity patterns between genotypes (Fig. 3-6B). During the first hour of the OF exposure, B6eGFPChAT and B6 genotypes each exhibit unbiased exploration of the OF (Fig. 3-6B; top row). During the last hour of analysis, B6 mice are found almost exclusively in the peripheral regions of the arena (Fig. 3-6B; bottom row). In contrast, B6eGFPChAT mice exhibited activity that was unbiased to either the peripheral or center regions of the OF. The pattern of activity and exploration by B6eGFPChAT mice was particularly evident during the last 20-minute interval (Fig. 3-6B; bottom row; purple). These data suggest that enhanced acetylcholine vesicular packaging may contribute to altered thigmotactic behaviour through increased activity and exploration to the novel environment.
Fig. 3-5. Novel environment locomotion and habituation in B6eGFPChAT mice. A: Horizontal beam break activity in a novel open field for B6eGFPChAT (N=11) and B6 control mice (N=9). B: Habituation to the novel open field measured as cumulative two hour horizontal activity for B6eGFPChAT (N=11) and B6 control mice (N=9). C: Rearing activity in a novel open field for B6eGFPChAT (N=11) and B6 control mice (N=9). As rearing events are registered, the mouse must go below the level of the vertical sensor for one second before the next rearing event can be recorded. D: Habituation to the novel open field measured as cumulative two hour rearing events for B6eGFPChAT (N=11) and B6 control mice (N=9). * p<0.05; ** p<0.01 compared to B6 controls. # p<0.05; ## p<0.01 compared to day 1.
3.4.7 B6eGFPChAT mice show increased activity in the dark/light box

To further characterize anxiety levels in B6eGFPChAT compared to B6 mice, the dark/light box paradigm was employed. The dark/light task is based on the innate aversion of mice to brightly lit areas and on the spontaneous exploration of mice in response to mild stressors, in this case novel open environments and light (Crawley, 2007). The aversion to the environment is measured by the time and total distance accumulated in each compartment. In this test, B6eGFPChAT and B6 control mice spent approximately 40% of their total distance (Fig. 3-7A) and time (Fig. 3-7B) in the light compartment and were found not to be significantly different from each other (Mann-Whitney U test = 42.00, P = 0.649 and Mann-Whitney U test = 39.00, P = 0.447 respectively).

Transitions between the light and dark compartments are considered an index of activity and exploration. In this study, the number of transitions was significantly greater for B6eGFPChAT compared to B6 control mice (Mann-Whitney U test = 21.50, P = 0.036) (Fig. 3-7D). Similarly, B6eGFPChAT mice accumulated a significantly greater total distance over the 10 minute duration than B6 controls (t(18) = 2.740; P = 0.013) (Fig. 3-7C). These results reiterate that B6eGFPChAT mice do not exhibit perturbed anxiety to open environments and light per se, however B6eGFPChAT mice are more active and display increased exploration to the novel environment of the dark/light box.

3.4.8 B6eGFPChAT mice exhibit enhanced exploration of the elevated plus maze

The EPM generates an approach/avoidance response using open, elevated arms to measure anxiety-like behaviour. Data from the EPM experiments revealed significant differences in the main parameter indicative of anxiety-like behaviour, namely the time spent in the open arm (t(18) = 2.150; P = 0.045) but not the number of open arm entries (Mann-Whitney U test = 36.00; P = 0.322) (Fig. 3-8A,B). Parameters reflecting changes in locomotor activity in this model were also found to be significantly different including the number of closed arm entries (Mann-Whitney U test = 16.50; P = 0.013) and total distance (t(14) = 2.150; P = 0.029) (Fig. 3-8B,C). These data revealed another aspect of the exploratory phenotype to novel environments in B6eGFPChAT mice, as these mice accumulated greater total distance and increased preference
Fig. 3-6. Open field anxiety in B6eGFPChAT mice. A: Proportion of total time spent in the center of a novel open field environment recorded in five minute intervals over two hours for B6eGFPChAT (N=11) and B6 control mice (N=9). B: Representative cumulative activity plots during the first hour (top row) and second hour (bottom row) for B6eGFPChAT and B6 control mice. Activity is presented in twenty minute intervals that have been color coded based on the discontinuous color legend.
to the open arm. The latency to enter the open arm was not used as an outcome measure here as mice were placed into the center of the maze facing one of the open arms.

3.5 Discussion

Here we present biochemical and behavioural characteristics of B6eGFPChAT mice that delineates the role of VACHT overexpression on cholinergic function, focusing on peripheral motor function, locomotion and anxiety. Our data provide evidence that modest increases in VACHT expression, previously associated with increased acetylcholine release (Nagy and Aubert, 2012), elicits physiological consequences, including spontaneous and novelty-induced locomotor activity. Collectively, these results provide insights on the importance of acetylcholine storage and release on behaviour, and this may have implications in human neurodegenerative disorders that exhibit cholinergic dysfunction.

3.5.1 Biochemical Analysis

We previously described that 3 month-old B6eGFPChAT mice have increased VACHT gene and protein expression that results from increased genomic copies of the cholinergic gene locus (Nagy and Aubert, 2012). These events are a consequence of the modified RP23-268L19 BAC, containing VACHT genomic sequence, that was used to initially generate the transgenic mice (Nagy and Aubert, 2012; Tallini et al., 2006). Increased VACHT expression enhanced acetylcholine release in the hippocampus (Nagy and Aubert, 2012), and likely enhanced cholinergic function in all brain regions where cholinergic terminals are found. Here, we found that VACHT overexpression is maintained at 6 months of age, spanning the age of animals used in this study. In contrast, no significant differences were found for ChAT and CHT protein expression, consistent with our and others previous findings that alteration in VACHT does not affect other presynaptic cholinergic proteins (Guzman et al., 2011; Nagy and Aubert, 2012). VACHT overexpression is therefore maintained at least up to 6 months in B6eGFPChAT mice without affecting ChAT and CHT expression.

3.5.2 Motor strength and coordination

Spontaneous and evoked release of acetylcholine at the neuromuscular junction is responsible for peripheral muscle contraction in response to motor neuron activation. As such, VACHT-
Fig. 3-7. Dark/light box test in B6eGFPChAT mice. A: Proportion of total distance accumulated in the lit portion of the open field in B6eGFPChAT (N=11) and B6 control mice (N=9). B: Proportion of total time accumulated in the lit portion of the open field in B6eGFPChAT (N=11) and B6 control mice (N=9). C: Total distance accumulated in the lit and dark portions of the open field in B6eGFPChAT (N=11) and B6 control mice (N=9). D: Total transitions between the light and dark portions of the open field in B6eGFPChAT (N=11) and B6 control mice (N=9). * p<0.05 compared to B6 controls.

Fig. 3-8. Elevated plus maze performance in B6eGFPChAT mice. A: Total time spent in the closed, center and open sections of the elevated plus maze in B6eGFPChAT (N=11) and B6 control mice (N=9). B: Number of entries into the open and closed arms of the elevated plus maze in B6eGFPChAT (N=11) and B6 control mice (N=9). * p<0.05 compared to B6 controls.
knockdown mice are significantly impaired in their ability to sustain prolonged physical activity, specifically in regards to forelimb grip strength, hanging endurance, and rotarod performance (de Castro et al., 2009b; Prado et al., 2006). An assessment of cholinergic tone at the neuromuscular junction has not been performed in B6eGFPChAT mice. The peripheral expression of the BAC transgene has been previously characterized in B6eGFPChAT mice (Tallini et al., 2006). Using the same mouse model, we found that VACHT is overexpressed in the CNS (Fig. 2-1) and peripheral regions of the autonomous nervous system (data not shown). Our analysis of neuromuscular function in B6eGFPChAT mice reveals that forelimb grip strength and ability to freely support their body weight using an endurance paradigm were maintained. Furthermore, rotarod performance using an accelerating rod to assess coordination, motor learning and endurance was essentially identical between genotypes. The maintenance of motor function in VACHT-overexpressing mice may reflect the tolerance that exists within the neuromuscular junction to withstand changes in cholinergic transmission. Under normal physiological conditions, peripheral cholinergic neurons maintain cholinergic function through readily releasable pools of acetylcholine-containing synaptic vesicles. During prolonged stimulation, large storage reserves of acetylcholine-containing vesicles can be localized within peripheral cholinergic neurons and used for synaptic release (Rizzoli and Betz, 2005). For these reasons, the impact of VACHT overexpression on neuromuscular function may require more demanding physical conditions to be resolved. Indeed, previous studies have identified that CHT overexpression improves performance during endurance treadmill paradigms, while CHT deficiency impaired treadmill performance (Bazalakova et al., 2007; Lund et al., 2010). It remains to be determined whether similar paradigms would elicit an effect in B6eGFPChAT mice.

In contrast to peripheral neurons, central cholinergic neurons have smaller pools of readily releasable vesicles, and as such may be more dependent on the rapid recycling of vesicles. Under certain physiological scenarios, such as when synaptic vesicles cycle faster than they can be filled (Prado et al., 2002), neurotransmitter transporters may be rate limiting to neurotransmitter release. During these events, the rate of acetylcholine release may be enhanced during VACHT overexpression and as such, central cholinergic functions may be more sensitive to modified levels of VACHT.
3.5.3 Spontaneous activity and circadian rhythms

Acetylcholine is known to play a complex role in the regulation of locomotor control, including acting as a modulator of the dopaminergic system (Drenan et al., 2010; Lester et al., 2010; Rice and Cragg, 2004; Threlfell et al., 2010). In particular, acetylcholine within the laterodorsal and pedunculopontine tegmental nuclei of the pons has been shown to mediate the dopaminergic activity along the nigrostriatal pathways whose innervation to the dorsal striatum is responsible for voluntary motor control (Lester et al., 2010). In addition, striatal cholinergic interneurons regulate dopamine release via beta2 subunit containing nAChRs present on dopaminergic axons in the striatum (Threlfell et al., 2010). Several reports show that pharmacological or genetic alteration of cholinergic or dopaminergic function leads to increased striatal dopamine release and increased spontaneous locomotion (Drenan et al., 2010; Giros et al., 1996; Gomeza et al., 1999; Rice and Cragg, 2004; Threlfell et al., 2010).

In addition to dopaminergic modulation of locomotion in the striatum, the contribution of forebrain cholinergic tone in spontaneous locomotion has recently been revealed. Mice with VAChT deficiency throughout the CNS and PNS (Martins-Silva et al., 2011) or specifically in basal forebrain neurons (Martyn et al., 2012) display hyperactivity. Interestingly, cholinergic contribution to locomotion appears to be independent of cholinergic striatal interneurons because selective removal of VAChT in the striatum does not induce hyperactivity (Guzman et al., 2011). It is therefore plausible that cholinergic innervation to other central regions, including the cortex and hippocampal formation, play important roles in the regulation of this behaviour.

Our findings that B6eGFPChAT mice exhibit hypoactive spontaneous activity are consistent with the notion that acetylcholine “turns down” neuronal circuits controlling spontaneous locomotion (Martins-Silva et al., 2011; Martyn et al., 2012). The observed hypoactivity in B6eGFPChAT mice was most evident during activity peaks occurring over the dark phase of the light-dark cycle. In addition, metabolic parameters of heat, VO$_2$ and CO$_2$ appear to correspond to daily rhythmic patterns of locomotion, with significant and corresponding decreases in VO$_2$ during the periods of significant hypoactivity. The transient decrease in VO$_2$ likely reflects the inherent decrease in respiration requirements associated with decreased activity. Taken together, these data suggest that the change in spontaneous activity is closely associated to the activity-rest pattern of B6eGFPChAT mice. These data are consistent with previous findings showing that
normal activity-rest patterns are regulated by cholinergic neurotransmission, potentially through β2*-nAChR of the suprachiasmatic nucleus (Liu and Gillette, 1996; Xu et al., 2011; Yang et al., 2010). This is because cholinergic neurotransmission is generally associated with a series of characteristic sleep changes, including decreased REM latency and increased REM density (Sarter and Bruno, 1999; Vazquez and Baghdoyan, 2001). As such, we considered that the sleeping patterns in B6eGFPChAT mice could have contributed to the observed patterns of activity in this study. However, this was found not to be the case, and when activity and inactivity were analyzed by determining movement by infrared beam break (Pack et al., 2007), no significant differences were found in the patterns of sleep time, sleep bout number or sleep bout duration were found. Collectively, these data suggest that VACHT overexpression induces generalized locomotor hypoactivity that is unrelated to circadian sleep regulation. VACHT overexpression in B6eGFPChAT mice has not been targeted to specific brain regions, limiting the identification of specific brain areas responsible for the observed hypoactivity. However, based on the discussion above, we postulate that VACHT overexpression is enhancing the inhibitory effect of acetylcholine via cholinergic basal forebrain or dopaminergic striatal networks. Indeed, the decreased spontaneous activity exhibited by B6eGFPChAT mice is reminiscent of mouse models with increased acetylcholine (via AChE inhibition) or decreased dopamine neurotransmission (Kobayashi et al., 1995; Zhou and Palmiter, 1995). Confirmation of these potential mechanisms awaits region-specific VACHT overexpression models.

3.5.4 Exploratory behaviour

Novel stimuli, including new or modified environments, generate approach/avoidance conflicts in mice. The conflict tests the balance between exploring the novelty to gain information and the anxiety-related cautiousness to avoid danger or harm. Exposure to novel stimuli has been extensively associated with cholinergic activation. Studies using exposure to novel environments and sensory stimulation as the experimental paradigms have also shown increased acetylcholine release in the nucleus accumbens, hippocampal formation and cortical structures (Giovannini et al., 2001; Schildein et al., 2000; Thiel et al., 1998). Furthermore, a number of studies have demonstrated that cortical (Day et al., 1991), striatal (Cohen et al., 2012) and hippocampal (Day et al., 1991; Dudar et al., 1979; Mizuno et al., 1991) acetylcholine release is positively correlated to behavioural arousal in novel environments as defined by locomotor activity. We therefore
investigated the exploratory behaviour in B6eGFPChAT mice in novel environments to evaluate the contribution of VACHT overexpression.

The results from the OF experiments indicate that B6eGFPChAT mice display transient increases in activity upon initial exposure to the novel environment compared to B6 control mice, including increased horizontal activity and rearing. These increased levels of exploration return to normal following the first 10 minutes of the OF exposure, where B6eGFPChAT mice begin to elicit normal intra-session patterns of habituation. Upon repeated exposure to the novel environment, B6eGFPChAT mice displayed only a modest decrease in locomotion, which did not reach significance, and was found to be significantly different than B6 control mice by day 3. The intra-session and inter-session habituation patterns of B6 control mice were found to be consistent with previous reports (Bolivar and Flaherty, 2003; Bolivar et al., 2000). While B6eGFPChAT mice intra-session habituation was unchanged, the impaired status of inter-session habituation in this study was unexpected. This is because earlier studies have shown that deficits in habituation are attributed to acetylcholine deficiency (Schildein et al., 2000; Schildein et al., 2002; Ukai et al., 1994), as acetylcholine levels in the hippocampus (Giovannini et al., 2001) or cortex (Sarter and Bruno, 1999; Sarter and Parikh, 2005) may contribute to memory consolidation or attention processes following exposure to the novel environment. We considered that inter-session habituation to novel environments may be the result of two components, one related to memory and anxiety and one related to motor activity. Indeed, previous experiments performing repeated exposures to novel environments reveal that during initial exposures, elevated acetylcholine released from cortical and hippocampal regions may be associated with fear, stress and motor activity (Giovannini et al., 2001). Subsequent habituated exposures have a limited component of memory and anxiety, as the inherent fear elicited by the novel of the environment is minimized, and as such cortical and hippocampal cholinergic activation is related primarily to motor activity (Giovannini et al., 2001). As such, we propose that the observed inter-session activity in B6eGFPChAT mice is attributed to increased exploration associated locomotion of B6eGFPChAT mice exposed to novel environments rather than impaired habituation per se. This speculation can be supported by the observed rearing habituation, which suggests that, to a certain extent, habituation behaviour is maintained in B6eGFPChAT. Furthermore, our observed locomotor arousal is consistent with the mechanism that instantaneous release of acetylcholine positively correlates with increased activity in novel
environments (Cohen et al., 2012; Day et al., 1991; Dudar et al., 1979; Mizuno et al., 1991), and suggests that VAChT overexpression may potentiate this response.

3.5.5 Anxiety-like behaviour

Endogenous cholinergic tone has been associated with anxiety-like behaviour in mice. The effect of acetylcholine is complex in that increased acetylcholine release has been associated with both anxiolytic and anxiogenic actions (File et al., 1998; File et al., 2000a). For this reason, the relationship between acetylcholine and anxiety may be related to regional subunit configurations of acetylcholine receptors in the CNS (File et al., 2000a; Gotti and Clementi, 2004; Labarca et al., 2001; McGranahan et al., 2011; Salas et al., 2003). In this study, we utilized multiple experimental paradigms (OF, DLB and EPM) known to elicit behavioural response in mice to assess the role of VAChT overexpression on anxiety-like behaviour.

When exposed to a novel OF, B6eGFPChAT mice did not show any center versus peripheral exploratory bias during the first five minutes of analysis, the time that has been previously shown to elicit the most robust anxiety behaviour, or over the entire duration of the assay. The strongly significant interaction that was observed during the OF exposure is clarified by considering the activity traces for the test. Whereas each genotype exhibits unbiased exploration of the OF during the first 60 minutes of analysis, B6eGFPChAT mice show dramatically more exploration in the OF compared to B6 control mice during the final 60 minutes of analysis. Consistent with these findings, the dark/light box did not differentiate between genotypes with respect to the primary outcomes of time and distance accumulation in the light field. However, an unbiased increase in total distance was revealed for B6eGFPChAT mice that are reflected by an increase in the total transitions between the dark and light fields. OF and DLB did not detect significantly anxiety-like differences between B6eGFPChAT and B6 control mice. However, B6eGFPChAT mice showed a moderate but significant bias to the open arms, suggesting that VAChT overexpression decreased anxiety-like behaviour in the EPM. The decreased anxiety-like behaviour observed in the EPM in the context of the released exploratory inhibition observed during each of the anxiety-like behavioural tasks suggests that the genetic modifications in the B6eGFPChAT have an anxiolytic effect. The divergent findings in the primary outcomes of the OF and DLB (no change in anxiety) and the EPM (decreased anxiety) can be reconciled as these tasks may not provide the same sensitivity as the EPM, which delivers a more complex
anxiogenic insult (Crawley, 2007). Alternatively, changes in the primary outcome of the EPM during VACht overexpression may be solely based on the modified exploratory locomotion in the B6eGFPChAT mouse.

3.5.6 Implications and concluding remarks

In the present study, we used congenic B6eGFPChAT mice that are homozygous for the RP23-268L19-EGFP transgene and have been previously characterized as having increased VACht gene and protein expression (Nagy and Aubert, 2012). These commercially available mice have been recently utilized during the investigation of multiple cholinergic pathways primarily for the identification and functional characterization of cholinergic neurons (Ade et al., 2011; Krasteva et al., 2011; Ogura et al., 2011; Rosas-Ballina et al., 2011). Here, we identified that B6eGFPChAT mice present a unique behavioural phenotype compared to B6 controls. While it remains possible that the observed phenotype will be confounded by positional effects related to the random insertion of the BAC transgene, only a single commercially available B6eGFPChAT founder exists precluding our examination using multiple founders with independent insertion sites. Keeping these limitations in mind, a cholinergic rationale related to the observed increase in VACht protein and previously defined enhancement in acetylcholine release (Nagy and Aubert, 2012) is congruent with the data and it provides a plausible explanation to the observed behaviour in B6eGFPChAT mice.

The utility of the B6eGFPChAT mouse as an experimental model for VACht overexpression could be of significance for future studies related to neurodegeneration. Significant decreases in VACht expression have been associated with various neurodegenerative conditions (Bell and Cuello, 2006; Bohnen and Albin, 2011; Chen et al., 2011b; Efange et al., 1997; Kuhl et al., 1996). Most notably, progressive VACht deficiency is observed during AD progression (Bell and Cuello, 2006; Chen et al., 2011b) and in post-mortem AD brains (Chen et al., 2011a; Chen et al., 2011b; Efange et al., 1997). Interestingly, the disease pathology of AD is also marked by abnormal motor behaviour including spontaneous hyperactivity and restlessness (Bedrosian et al., 2011; Mega et al., 1999; Ognibene et al., 2005; Sterniczuk et al., 2010b; Walker et al., 2011), as well as enhanced anxiety to novelty (Bedrosian et al., 2011; Sterniczuk et al., 2010a). The series of experiments described in this study suggest that increased VACht expression observed in B6eGFPChAT mice contributes to spontaneous hypoactive behaviour and increased
exploration in novel environments. In cases of cholinergic deficiency and impaired locomotor-related behaviour, identifying approaches to upregulate VACHT may be of therapeutic significance.
Chapter 4
Hypercholinergic mice exhibit enhanced dendritic complexity from newborn neurons and improved spatial memory during aging

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P.M.N. led and conducted all experiments and drafted the manuscript. I.A. provided materials, analysis tools and experimental design as well as manuscript revision.
4.1 Abstract

Aging is marked by progressive impairments in the process of adult neurogenesis and spatial memory performance. The underlying mechanisms for these impairments have not been fully established, however they may coincide with decline in cholinergic signalling in the hippocampus. This study investigates whether augmenting cholinergic neurotransmission, by enhancing the expression of the vesicular acetylcholine transporter (VACHT), influences the age-related decline in the development of newborn hippocampal cells and spatial memory. We found that enhanced VACHT expression in the hippocampus of mice contributes to lifelong increases in the dendritic complexity of newborn neurons. Furthermore, enhanced VACHT expression improved memory acquisition through increased use of spatially precise search strategies in the Morris water maze through the course of the aging process. These data suggest that VACHT overexpression leads to increases in dendritic plasticity and provides resilience to aged-related deficits in spatial memory.

4.2 Introduction

The production of newborn neurons persists into adulthood in selected regions of the brain, including the subventricular zone of the lateral ventricles and the subgranular zone of the dentate gyrus. In the dentate gyrus, proliferating cells along the subgranular zone undergo critical stages of migration to the granule cell layer and maturation, including the formation of dendritic processes that project to the molecular layer. Ultimately, newborn neurons integrate into the pre-existing trisynaptic circuit, which receives input from the entorhinal cortex and provides output to the cornu ammonis regions of the hippocampus.

Adult neurogenesis has been linked to the role of the hippocampus in modulating spatial memory in both humans and rodents (Garthe and Kempermann, 2013; Ming and Song, 2011). Newborn hippocampal neurons appear to contribute to these processes through unique physiological properties that make them susceptible to behavioural-dependant synaptic plasticity. Congruent to this hypothesis are rodent studies that have shown hippocampal neurogenesis to be modulated by behavioural activity (Ambrogini et al., 2000; Gould et al., 1999a; Hairston et al., 2005; Kempermann et al., 1997). In addition, spatial memory improvement correlates with increased
neurogenesis (Van Praag et al., 1999; Van Praag et al., 2005; Wang et al., 2012) and is impaired following a loss of hippocampal neurogenic function (Rola et al., 2004; Snyder et al., 2005).

Despite neurogenesis persisting into adulthood, continued aging is associated with dramatic reductions in cell proliferation (Bondolfi et al., 2004; Gil-Mohapel et al., 2013; Kempermann et al., 1998; Kuhn et al., 1996), the number of immature neurons (Gil-Mohapel et al., 2013; Kuhn et al., 1996) and a retraction of dendritic morphology. In parallel with these findings, many aspects of spatial memory performance show age-related decline (reviewed in Woodruff-Pak et al., 2011) suggesting a causal relationship with the reduction of newborn neurons. Substantial evidence suggests that cholinergic neurotransmission regulates adult neurogenesis, and a depletion of cholinergic input to the neurogenic milieu during the aging process may contribute to spatial memory deficits (Cooper-Kuhn et al., 2004; Kaneko et al., 2006; Mohapel et al., 2005; Schliebs and Arendt, 2006). For example, cholinergic lesions decrease (Cooper-Kuhn et al., 2004), while acute enhancement of acetylcholine increases (Kaneko et al., 2006), the survival of newborn neurons. Chronic nicotine exposure reduces the proliferation adult born neurons (Scerri et al., 2006; Shingo and Kito, 2005) while nicotinic acetylcholine receptor (nAChR) deficient mice exhibit decreased proliferation (Campbell et al., 2010; Harrist et al., 2004) and a truncated dendritic network (Campbell et al., 2010).

Here we evaluated whether the overexpression of the vesicular acetylcholine transporter (VACHT) can influence the development of newborn neurons in the hippocampus and enhance spatial memory. We used the B6eGFPChAT mouse model with increased hippocampal acetylcholine release due to VACHT overexpression (Nagy and Aubert, 2012; Nagy and Aubert, 2013). We demonstrate that VACHT overexpression significantly enhanced the dendritic complexity of the immature neuronal network across aging in B6eGFPChAT mice. Furthermore, aged B6eGFPChAT mice exhibit improved spatial memory acquisition, which was related to the use of spatially precise search strategies in the Morris water maze (MWM). These findings support the role of cholinergic signalling in adult neurogenesis, spatial memory consolidation and provide VACHT as a sufficient target to enhance cholinergic functions.
4.3 Materials and Methods

4.3.1 Animals

B6 mice (Jackson Laboratories, Bar Harbour, ME, USA) and B6 mice containing homozygous congenic copies of the RP23-268L19-EGFP transgene (B6eGFPCbAT; Jackson Laboratories, Bar Harbour, ME, USA) were used in this study at 4-, 6-, 18- and 24-months of age (Nagy and Aubert, 2012). B6eGFPCbAT mice exhibit increased expression of VACHT leading to increased acetylcholine release in the hippocampal formation (Nagy and Aubert, 2012). Presence of the transgene was confirmed using conventional PCR and primers as previously described (Tallini et al., 2006), and by the expression of eGFP observed during immunofluorescence microscopy protocols. All animal protocols were approved by the Animal Care Committees of Sunnybrook Research Institute and the University of Western Ontario, and were performed according to the guidelines set by the Canadian Council on Animal Care and the Animals for Research Act of Ontario.

4.3.2 Cell proliferation

B6 and B6eGFPCbAT mice at 4-months (N = 5/6), 6-months (N = 6/5) and 24-months (N = 4/5) of age received three injections of BrdU (50 mg/kg intraperitoneal, Sigma-Aldrich, St. Louis, MO, USA) on a single day spaced two-hours apart. Seven days later, mice were concurrently anaesthetized with a mixture of ketamine (75 mg/kg) and xylazine (10 mg/kg). The mice were then perfused intracardially with saline, followed by fixation with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed, post-fixed overnight and equilibrated in 30% sucrose. Coronal sections were cut at 40 µm and collected in 96-well plates filled with cryoprotectant solution (50 mM phosphate buffer; 25% (v/v) glycerin; 30% (v/v) ethylene glycol; pH 7.4). All incubations were carried out at room temperature with gentle agitation unless otherwise noted. Antigen retrieval was performed using 2 N hydrochloric acid for 30 min at 37°C, followed by neutralization to pH 8.5 in borate buffer. Sections were rinsed 3x10 min in PBS and blocked for 1 h using PBS containing 5% donkey serum and 0.3% Triton X-100 (PBS++). Sections were incubated in PBS++ containing antibodies against BrdU (1:400; AbD Serotec, Raleigh, NC, USA) overnight at 4°C. Sections were rinsed 3x10 min in PBS and incubated in PBS++ containing the appropriate CY3-conjugated secondary antibody (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h. Sections were rinsed and
incubated in PBS++ containing antibodies against Ki67 that were preconjugated to Alexa 647 fluorophores (1:50; New England Biolabs, Ipswich, MA, USA) overnight at 4°C. For 24-month old tissue, sections were incubated in 0.1% Sudan black for 10 mins to reduce lipofuscin autofluorescence. Sections were then rinsed and mounted on slides for imaging. Fluorescent labelling was detected by confocal microscopy (Zeiss Observer Z1, Yokogawa CSU-X1; Carl Zeiss, Don Mills, Canada). Cells positively labeled with antibodies against BrdU or Ki67 were counted in bilateral sections at a 1:12 interval, along the SGZ of the dorsal DG.

4.3.3  Dendritic maturation

B6 and B6eGFPChAT mice at 4-months (N = 5/6), 6-months (N = 6/5) and 24-months (N = 4/5) of age were sacrificed and perfused as previously described. 40 μm coronal sections were blocked in PBS containing 2% donkey serum and 0.3% Triton X-100 (PBS++) for 1hr followed by an overnight incubation in PBS++ containing antibodies against DCX (1:200; Santa Cruz, Dallas, TX, USA). Sections were rinsed and incubated in PBS++ containing the appropriate CY5-conjugated secondary antibody. Z-stacks of DCX-positive cells were imaged using confocal microscopy (Zeiss Axiovert 100M, LSM 510; Carl Zeiss, Don Mills, Canada) and quantified in unilateral sections at a 1:12 interval along the SGZ of the dorsal DG. Dendrites were chosen based on vertical orientation in the granular cell layer and traced using the Simple Neurite Tracer plugin of FIJI (Schindelin et al., 2012). Three-dimensional dendritic branching was measured using Sholl analysis at radial increments of 1μm.

4.3.4  Morris Water Maze

B6 and B6eGFPChAT mice were exposed to the MWM at 4-months (N = 9/11), 18-months (N = 9/10) and 24-months (N = 7/8) of age using a procedure as previously described (Vorhees and Williams, 2006). Briefly, mice received four consecutive training trials, during which the hidden platform was kept in a constant location with a 15-minute inter-test interval. Mice were placed at a different starting location within the pool for each trial, which consisted of a swim followed by a 10-second platform sit. Spatial acquisition was assessed during the set of four trials per day for four consecutive days. Spatial retrieval was assessed using a 1-min probe trial on the fifth day with the hidden platform removed (Vorhees and Williams, 2006). All data were recorded using a
video camera and analyzed using ANY-Maze video tracking software (Stoelting Co., Wood Dale, IL, USA).

The parameters used to assess learning included latency to reach the platform (escape latency), distance to reach the platform (escape distance), average swim speed during the trial (swim speed) and the search strategy used during each trial. The predominant search strategy for each trial was objectively classified using a criterion-based algorithm as follows (Garthe et al., 2009; Gil-Mohapel et al., 2013): (1) direct swim, characterized by a maintained heading towards the platform (mean heading <20° away from the platform); (2) focal search, characterized by a highly localized search near the platform (≥50% trial in 15 cm radius target zone, centered on the platform location); (3) directed search, characterized by a preference for a corridor towards the platform or platform quadrant (>80% trial within a 50 cm-wide corridor from start point to platform); (4) chaining, characterized by searching near the correct radial distance of the platform to the wall (>75% trial 20–50 cm from pool center, <15% within 10 cm of wall, and <10% within 20 cm of wall); (5) scanning, characterized by a preference for the central pool area in which distal cue visibility is maximal (>50% trial within 35 cm of pool centre); (6) thigmotaxis, characterized by maintaining close proximity to the wall (>70% trial within 10 cm of wall); (7) perseverance, characterized by maintaining close proximity to the wall (>70% trial within 10 cm of wall); (8) random search, characterized by no other classified strategy (remaining unclassified trials). Probe trial performance was assessed by the amount of time spent in the target quadrant (the previous location of the escape platform) along with the left and right adjacent and opposite quadrant to the target, as well as the number of platform crossings and the total distance accumulated during the trial.

4.3.5 Statistical Analysis

Data are represented either as individual cases or as the mean ± SEM. Cell counts and Sholl crossings were examined using two-way ANOVA with Bonferroni post hoc analysis when main effects were found. MWM spatial acquisition parameters were assessed and compared within ages using repeated measures ANOVA with Bonferroni post hoc analysis. MWM spatial strategy frequencies were compared across trials within each age group using Friedman’s ANOVA, whereas comparisons among age groups were assessed using Spearman Rank Order Correlations. MWM probe parameters were assessed using one-way ANOVA and Dunnett’s post
hoc analysis for comparisons of the time each genotype spent in the target quadrant versus the remaining quadrants and two-way ANOVA to compare parameters between genotypes. P<0.05 was considered statistically significant. Statistical outcomes of main effects are fully described in the text. Statistical outcome of post-hoc analysis are indicated in the figures.

4.4 Results

4.4.1 Cell proliferation of newborn hippocampal neurons

Ki67 and BrdU immunohistochemistry was used to label proliferating cells in the subgranular zone of the dentate gyrus, and the 7-day survival of newborn granule cells respectively (Fig. 4-1A, B). Counts of the number of Ki67-positive cells in the SGZ-DG revealed a significant age factor \( [F_{(2,25)} = 153.60, \text{P}<0.001] \) and a trend towards a genotype factor \( [F_{(1,25)} = 4.04, \text{P}=0.05] \) (Fig. 4-1C). A significant interaction between age and genotype was also revealed \( [F_{(1,25)} = 12.96, \text{P}<0.001] \) (Fig. 4-1C). Similar to Ki67, counts of the number of 7-day old BrdU-positive cells in the DG revealed a significant age factor \( [F_{(2,25)} = 144.50, \text{P}<0.001] \) with a trending genotype factor \( [F_{(1,25)} = 4.14, \text{P}=0.06] \) (Fig. 4-1D), reinforcing the impact of VAChT overexpression on the proliferation of newborn granule cells the dentate gyrus. The age-dependant declines observed for the numbers of Ki67-positive (proliferating) and BrdU-positive (7-day old) cells suggest that the 7 day survival of these newborn neurons is not significantly impacted by VAChT overexpression from 4- to 24-months of age (Fig. 4-1C,D).

4.4.2 Dendritic Complexity of newborn hippocampal neurons

DCX immunohistochemistry was used to identify newly differentiated neurons in the dentate gyrus of the dorsal hippocampus (Fig. 4-2). Analysis of DCX-positive cells revealed a significant age factor \( [F_{(1,25)} = 89.89, \text{P}<0.001] \) (Fig. 4-2A); however, no significant difference in the number of newborn neurons between genotypes at 4-, 6- or 24-months of age were seen, indicating that the decrease in newborn neurons during is unaffected by VAChT overexpression (Fig. 4-2A). Sholl analysis using dendritic tracings of DCX-positive fibers (Fig. 4-2B) was used to quantify the complexity of dendritic branching as a function of increased distance from the cell soma. At 4-, 6- and 24-months of age, B6eGFPChAT mice exhibited enhanced dendritic complexity compared to B6 controls [4-months: \( F_{(1,269393)} = 710.80, \text{P}<0.001; \) 6-months: \( F_{(1,65746)} = 313.00, \text{P}<0.001; \) 24-month: \( F_{(1,3435)} = 9.14, \text{P}<0.01 \) (Fig. 4-2C-E). A significant
Fig. 4-1. VACHT overexpression does not impact hippocampal cell proliferation during aging. (A) Schematic for proliferation and survival study. (B) Ki67-positive (blue, filled arrows) and BrdU-positive (red, open arrows) cells in the granule cell layer of the dentate gyrus (DG-GCL) in 4-month old mice. (C-D) No significant differences in age-related decline of the number of Ki67-positive cells in the DG-GCL (C) or BrdU-positive cells in the DG (D) were found between genotypes. †††P<0.001 (ANOVA age effect). Scale bar = 25 µm. Abbreviations: BrdU, 5-bromo-2'-deoxyuridine; d, day; DG, dentate gyrus; DG-GCL, dentate gyrus and granule cell layer; DG-SGZ, subgranular zone of the dentate gyrus; SAC, sacrifice; tid, ter in die.
age effect was observed for the average maximum dendritic length \( F(2,25) = 83.13, P<0.0001 \) however no genotype effect was detected (data not shown). Taken together, these data demonstrate that VACChT overexpression increases the dendritic complexity of immature newborn neurons.

4.4.3 Morris water maze: Age-associated changes in spatial learning

To assess the effect of enhanced VACChT expression on spatial learning, B6 and B6eGFPChAT mice were trained to locate the position of a hidden escape platform in the MWM at 4-, 18- and 24-months of age. Significant trial effects were observed at each age for escape latency [4-months: \( F(1,18) = 72.04, p<0.001 \); 18-months: \( F(1,18) = 41.77, P<0.001 \); 24-month: \( F(1,18) = 44.78, P<0.01 \)] and total distance [4-months: \( F(1,18) = 72.25, P<0.001 \); 18-months: \( F(1,18) = 40.90, P<0.01 \); 24-month: \( F(1,18) = 54.50, P<0.001 \)] (Fig. 4-3A-C), suggesting that the animals were able to learn the task independent of their age. At 24-months of age, significant genotype factors were revealed for total distance [\( F(1,18) = 7.34, P<0.01 \)] and average speed [\( F(1,18) = 29.92, P<0.01 \)] (Fig. 4-3B,C). These data suggest while the time taken discover the hidden platform is equal between genotypes, B6eGFPChAT swim a significantly shorter distance in that time compared to B6 controls.

The use of spatially precise and imprecise search strategies has been shown to contribute to differences in escape distance and latency. Given that we observed a decrease in escape distance but not latency in B6eGFPChAT mice (Fig. 4-3B,A; respectively), we considered the possible preference for more spatially precise search strategies. A detailed analysis of the predominant search strategy used in each of the four days at each age was performed using a criterion-based algorithm (Fig. 4-4A). At the beginning of training (day 1), spatially neutral and spatially imprecise (mainly random search and perseverance) predominated for each genotype (Fig. 4-4B,D). However, for each age group, there was a shift to increased use of spatially neutral and spatially precise (mainly direct search, chaining, scanning) strategies throughout the four day training period in B6 [4-months: \( 4(7) = 17.63, P=0.014 \); 18-months: \( 4(7) = 24.80, P=0.001 \); 24-months: \( 4(7) = 18.07, P=0.012 \)] and B6eGFPChAT mice [4 months: \( 4(7) = 18.11, P=0.011 \); 18-months: \( 4(7) = 24.24, P=0.001 \); 24-months: \( 4(7) = 23.36, P=0.001 \)]. Spearman’s rank order correlation revealed that for B6 controls at all ages, significant decreases in the frequency of
Fig. 4-2. VACHt overexpression enhances dendritic complexity in newborn hippocampal neurons. (A) No significant differences in the age-related decline of DCX-positive cells number were found between genotypes. (B) Representative tracings of DCX-positive dendrites in 4-month old mice. (C-E) Dendritic complexity arising from newborn neurons were enhanced in B6eGFPChAT mice at 4-, 6- and 24-months of age compared to controls. *P<0.05; **P<0.01; ***P<0.001 (Bonferroni post-hoc analysis); †††P<0.001 (ANOVA age effect). Abbreviations: DCX, doublecortin; GCL, granule cell layer; MCL, molecular cell layer; SEM, standard error of the mean; SGZ, subgranular zone.
perseverance [P<0.05] and random search [P<0.01] were matched by increases in scanning
[P<0.01] and chaining [P<0.01] with training (Fig. 4-4B). B6eGFPChAT mice exhibited a
significant decrease in random search [P<0.01] and an increase in chaining [P<0.01] with
training (Fig. 4-4C). Spearman’s rank order correlation also revealed a significant decrease in the
frequency of the random search strategy in B6eGFPChAT mice [P<0.01] with age (Fig. 4-4C).
In particular, while no significant age-dependent differences were observed in B6 controls (Fig.
4-4D), a reduction of the use in random search was observed in aged B6eGFPChAT mice when
compared to young B6eGFPChAT mice, matched by the alternative use of scanning, chaining
and direct search (Fig. 4-4E). From these data, VACHT overexpression appears to improve
spatial acquisition in aged mice, which is attributed to spatially precise search strategies.

4.4.4 Morris water maze: Age-associated changes in spatial memory

The total time spent in the target quadrant of the pool (where the platform used to be located)
during the 60-second probe trial was measured compared to the remaining three quadrants (left
adjacent, right adjacent and opposite) (Fig. 4-5A-C). At 4-, 18- and 24-months of age,
preferences for exploration of the target quadrant compared to the adjacent and opposite
quadrants in B6 [4-months: F(3,35) = 13.63, P<0.001; 18-months: F(3,35) = 5.22, P<0.01; 24-
months: F(3,27) = 13.26, P<0.001] and B6eGFPChAT mice [4-months: F(3,43) = 42.85, P<0.001;
18-months: F(3,39) = 46.56, P<0.001; 24-months: F(3,31) = 22.23, P<0.001] were observed (Fig.
4-5A-C). Post-hoc analysis using Dunnett’s multiple comparison test revealed a significant
increase in time spent in the target quadrant compared to all other quadrants in all the mice, with
the exception of 18-month B6 mice (Fig. 4-5A-C). The time spent in the remaining quadrants fell
below the threshold of chance (25% of the trial; 15s). In 18-month B6 mice, the preference for
target was compared only to the opposite quadrant (Fig. 4-5B). Representative swim plots are
presented at 4-, 18- and 24-months of age for each genotype.

When comparing the percent time spent in the target quadrant, no genotype or age effects were
observed (Fig. 4-5D) indicating that neither genotype nor age affected the ability of the mice to
recall the prior location of the hidden target. The evaluation of target site crossing (the previous
location of the hidden platform) also revealed the absence of a genotype effect; however, a
significant age effect was observed [F(2,48) = 11.80, P<0.001] suggesting that repeated exposure
Fig. 4-3. 24-month old B6eGFPChAT mice exhibit shorter distance to escape and slower speed during MWM training. The four-trial mean values for escape latency (A), total distance to escape (B) and average speed (C) were plotted for each day of MWM training at 4-, 18- and 24-months of age. During day 3 and 4, 24-month old B6eGFPChAT mice accumulated a significantly shorter distance to escape and slower swim speed compared to controls. *P<0.05; **P<0.01 (Bonferroni post-hoc analysis); †P<0.05, ††P<0.01, †††P<0.001 (ANOVA age effect). Abbreviations: D1-4, Day 1-4; m, meters; sec, seconds; SEM, standard error of the mean.
Fig. 4-4. 24-month old B6eGFPChAT mice utilize more spatially precise search strategies during MWM training. (A) Representative examples of the eight distinct search strategies employed during training in the MWM that were recognized by the classification algorithm. Strategies are color coded. (B, C) Percentage of trials classified according to each search strategy across training days (Fig. 4-3) for the various age groups in B6 (B) and B6eGFPChAT (C) mice. See text for statistical comparisons. All groups showed a progression towards an increase in the use of hippocampal-dependent spatially precise strategies with training, particularly through increased chaining and decreased random search (P<0.05; P<0.01). B6eGFPChAT mice revealed a significant reduction in the use of random search (imprecise search strategy, P<0.01). (D, E) Percentage difference in the frequency of each search strategy in 24- compared to the 4-month old mice reveal increased use of scanning, chaining and direct search in B6eGFPChAT mice during aging. Abbreviations: D1-4, Day 1-4.
enhances the precision of the time spent in the target quadrant (Fig. 4-5E). Finally, to address the possibility that the increased target site crossing in aged mice was due to an increase in activity, the total distance accumulated during the probe trial was quantified (Fig. 4-5F). Using the two-way ANOVA, we detected an age effect \([F_{(2,48)} = 11.80, P<0.001]\) in the reduction of total distance during aging in both genotypes. Thus, while VACChT overexpression improves spatial acquisition, it did not contribute to a unique phenotype related to spatial memory during aging.

4.5 Discussion

In previous studies, we have shown that VACChT overexpression leads to increased acetylcholine release in the hippocampus (Nagy and Aubert, 2012) that is likely to be maintained throughout the life of the animal (Nagy and Aubert, 2013). In normal rodents, naturally occurring aged-related impairments in both the maturation of immature hippocampal neurons (Amrein et al., 2004; Ben Abdallah et al., 2010; Morgenstern et al., 2008), and hippocampal-dependant forms of spatial memory have been explored (Kennard and Woodruff-Pak, 2011). Here, we have characterized the effect of VACChT overexpression on cell proliferation and early neuronal maturation in the dentate gyrus and spatial memory during normal aging. We have shown that VACChT overexpression increases dendritic complexity of newborn neurons in the dentate gyrus without having an effect on their number or short-term survival. Increases in dendritic complexity in VACChT overexpressing mice were evident at 4-, 6- and 24-months of age, suggesting this effect to be independent of the normal age-related reduction of cell proliferation. In addition, we have shown that VACChT overexpression contributes to spatial memory performance attributed to the use of precise spatial navigation strategies in the MWM.

Cholinergic signalling has been implicated in select aspects of adult neurogenesis. Lesions of the basal forebrain, reducing the cholinergic contribution of the basal forebrain to the hippocampus, have been shown to decrease cell proliferation in the dentate gyrus (Cooper-Kuhn et al., 2004). However, the effect of elevated levels of acetylcholine is less clear. Acute increase of acetylcholine released to the hippocampus, augmented through the use of cholinergic agonists including AChE inhibitors, supports adult neurogenesis by increasing the proliferation and survival of newly formed neural progenitors (Berger et al., 1998; Cohen et al., 2008; Kaneko et al., 2006; Ma et al., 2000). In contrast, chronic nicotine exposure appears to
Fig. 4-5. B6eGFPChAT mice exhibit normal memory retrieval in the MWM. (A-C) The percentage of time spent in each quadrant of the maze was measured on day 5 in a 60-second probe trial with the platform removed at 4-, 18- and 24-months of age. Representative swim path plots for B6 (left; white field) and B6eGFPChAT (right; grey field) mice at 4-, 18- and 24-months of age. The dotted line indicates the chance threshold, set at 25% of the duration of the trial. (D) The percent time spent in the target quadrant was not significantly different between genotypes during aging. (E) There was no significant difference between genotypes in the improvement of platform crossing during aging. (F) There was no significant difference between genotypes in the decrease of total distance during aging. **P < 0.01; ***P < 0.001 (Dunnett’s post-hoc analysis); ††P < 0.01, †††P < 0.001 (ANOVA age effect). Abbreviations: L, left adjacent to the target; O, opposite to the target; R, right adjacent to the target; T, target; m, meters; sec, seconds; SEM, standard error of the mean.
decrease the proliferation of neural progenitor cells (Scerri et al., 2006; Shingo and Kito, 2005). Our report indicates that increased VACHT expression, with its associated acetylcholine vesicular packaging and release, has minimal effect on three early stages of new neuron production in the dentate gyrus during aging, specifically cell proliferation, short-term survival and neuronal fate-determination (i.e. DCX-immunoreactivity). These findings arise from lifelong VACHT overexpression and the analysis of age-related trends in cell numbers, in contrast to the relatively acute increases of cholinergic neurotransmission at the discrete ages previously studied. In addition, in our study, the release of acetylcholine remains governed by the endogenous cholinergic promoter, and subjected to normal levels of AChE activity at the synaptic cleft (Nagy and Aubert, 2012; Tallini et al., 2006). This strategy may preserve a better temporal element of cholinergic neurotransmission and post-synaptic activation, as compared to the application of AChE inhibitors or post-synaptic cholinergic agonists (i.e. nicotine). Under our conditions, enhanced acetylcholine does not appear to affect the proliferation of newborn hippocampal cells.

Dendritic maturation follows a sequential process of generation, elongation and retraction of dendritic branches resulting from intrinsic genetic programs and external modulators. Cholinergic signalling has been shown to contribute to the dendritic expansion of target neurons, including cortical pyramidal neurons that receive cholinergic input from the nucleus basalis (Hohmann et al., 1991). The role of acetylcholine appears to be instrumental in the time schedule of nascent neuron differentiation. For example, during the early development of immature hippocampal neurons, gamma-aminobutyric acid (GABA) input switches from depolarizing (excitatory) to hyperpolarizing (inhibitory) (Ming and Song, 2011). Neuromodulation of this switch involves acetylcholine, and appears to regulate dendritic maturation and integration (Campbell et al., 2010; Liu et al., 2006). Specifically, in mice that lack β2- or a7-containing nAChRs, newborn neurons are generated with decreased dendritic complexity and immature (i.e. depolarizing) GABAergic activity (Campbell et al., 2010; Lozada et al., 2012; Morley and Mervis, 2013). Immature neurons in the dentate gyrus also have been shown to express acetylcholine receptors directly (Kaneko et al., 2006), however any direct regulation by cholinergic input on dendritic maturation has not yet been established. Taken together, our report
of enhanced dendritic complexity in VACHT overexpressing mice throughout aging supports a role for cholinergic input in the dentate gyrus for the maturation of adult born neurons.

The dendritic network arising from newborn granule cells form functional circuits (Vivar and Van Praag, 2013) that contribute to the formation of spatial memories (Ming and Song, 2011). These actions may be mediated through their enhanced excitability and lower threshold for long term potentiation (Wang et al., 2000) or proposed roles in the generation of allocentric spatial maps (Clelland et al., 2009; Garthe et al., 2009). The MWM has been used extensively to evaluate hippocampal function relating to spatial cognition. Performance in the MWM is significantly reduced by 18-months of age (reviewed in Woodruff-Pak, 2011). In this study, we evaluated MWM performance using a longitudinal design, where previous training experience could contribute to the performance of subsequent exposures (Vicens et al., 2002), and may explain that deficiencies were not detected until 24-months of age. VACHT overexpression was shown to enhance MWM learning as exhibited by significantly reduced escape distance. Given that average speed was also significantly decreased in VACHT overexpressing mice, with no effect related to escape latency, we hypothesized that VACHT overexpressing mice use more spatially precise swim paths than aged controls. Indeed, previous studies show that enhanced adult neurogenesis may contribute to spatial precision in the MWM without an effect on escape latency (Garthe et al., 2009). As such, we evaluated the spatial strategies used to locate the platform using a criterion-based algorithm. We found that, consistent with previous reports, the use of more precise spatial strategies accompanied training at each age evaluated, in both genotypes. However, in contrast to B6 controls, B6eGFPChAT mice exhibited a statistically significant decline in the use of random search during aging. By specifically comparing strategies used at 24-months of age compared to those used at 4-months of age, B6eGFPChAT mice appeared to be more effective at employing spatially precise strategies compared to 4-month mice. Specifically, B6eGFPChAT mice use scanning and chaining strategies instead of random search, which we hypothesize to contribute to their reduced escape distance during MWM learning. Improved spatial precision in the MWM requires the integration of distal cues into a representation of the space, a specific function of the DG (Goodrich-Hunsaker et al., 2008). Garthe et al. (2009) previously proposed that newborn granule cells may therefore contribute to this process through the rapid encoding and integration of distal cue information when the context has changed In our study, the use of multiple MWM starting positions, held
constant within days supports the further formation of stable encoding rules for redundant stimulus configurations. We therefore propose that increases in dendritic complexity, in the absence of changes in granule cell proliferation and immature neuron cell number, may contribute to the formation of allocentric spatial representations in the hippocampus and ultimately improve spatial precision during acquisition. One must consider however that enhanced dendritic ramification is only one measured outcome within B6eGFPChAT mice and that other mechanisms, sensitive to increased cholinergic tone, may also contribute to improved MWM performance.

Despite the improved performance in spatial acquisition, spatial memory retrieval was maintained in aged B6eGFPChAT mice. The maintenance of spatial memory retrieval coincide with data reported by Martyn et al. (2012) on the reciprocal role of basal forebrain VACChT-depletion in the impairment of spatial acquisition but not spatial memory using the MWM paradigm. In addition, maintained spatial memory is consistent with the more general role of acetylcholine in the encoding, versus retrieval, of spatial memory (Barry et al., 2012). These findings do stand in contrast to a recent report using ChR2-ChAT-EYFP mice which also exhibit increased VACChT expression, but decreased MWM probe trial performance (Kolisnyk et al., 2013). While an explanation for this difference is not immediately clear, the number of VACChT copies (fifty compared to three in our model), and the potential for altered oxoglutarate dehydrogenase activity in ChR2-ChAT-EYFP mice, makes direct comparison difficult. In our hands, using B6eGFPChAT mice, the data suggest that despite differences in the rate of memory acquisition, VACChT overexpression does not augment the ultimate recall of the target location. In both genotypes, the percent time in the target quadrant during the MWM probe trial was not found to be dependent of age, consistent with previous longitudinal MWM analysis in B6 mice (Wong and Brown, 2007). It is also worth noting that we did observe an effect of the longitudinal design through an improvement in the number of target site crossings during aging. This fact would support the notion (Vicens et al., 2002; Wong and Brown, 2007) that previous exposures to the MWM may mitigate some of the normal age-related decline observed in B6 mice (Kennard and Woodruff-Pak, 2011). These parameters were not affected by VACChT overexpression.
In conclusion, increased complexity of newborn hippocampal dendrites were observed in B6eGFPChAT mice at 4-, 6- and 24-months of age, which was associated with enhanced memory acquisition in 24-month B6eGFPChAT mice compared to controls. Under our longitudinal evaluation of spatial memory in the MWM, the age-dependency of memory impairment appears during the acquisition of new information, and the contribution of cholinergic innervation is limited to the augmentation of spatial acquisition in aged animals. Future work could explore the possible relationships between the observed improvement in spatial acquisition and enhanced hippocampal dendritic complexity revealed in B6eGFPChAT mice. Models of enhanced VACHT expression specific to the septohippocampal pathway would be of interest to support the contribution of elevated hippocampal acetylcholine release to dendritic complexity of newborn neurons of the dentate gyrus and improved spatial acquisition during aging.
Chapter 5
General Discussion

5.1 Summary of Research Findings

This thesis focused on the regulation of cholinergic activity by VACHT. The underlying goal of this work was to evaluate whether VACHT acts as a sufficient target to modulate cholinergic neurotransmission and function. Previous work using VACHT-deficient animals revealed that reducing VACHT decreases the amount of acetylcholine released from cholinergic terminals and has demonstrated a wide variety of physiological and behavioural consequences. Our studies tested the possibility that VACHT can be used to increase acetylcholine release and that these changes would modulate behaviour related to cholinergic tone.

In Chapter 2, we describe the B6eGFPChAT mouse model, with an enhanced gene copy number of VACHT. Through the use of this model, it was demonstrated for the first time that elevations in VACHT protein lead to enhanced acetylcholine release from hippocampal slices. In Chapter 3, the consequence of VACHT overexpression was evaluated through multiple metabolic and behavioural paradigms. VACHT-induced acetylcholine release was found to modulate locomotion, anxiety and response to novelty. Finally, in Chapter 4, the ability of VACHT overexpression to counteract the age-related decline in hippocampal function was evaluated. VACHT-induced acetylcholine release was demonstrated to produce livelong enhancement in the dendritic branching of adult-born hippocampal neurons, and improvement of spatial navigation in aged animals.

The data presented in this thesis support the hypothesis that VACHT overexpression throughout the aging process mediates increases in acetylcholine release, improving cholinergic tone and related hippocampal plasticity and spatial cognition. The findings of this work highlight VACHT as an important regulator of cholinergic tone, neuronal plasticity and cognitive function, which may offer novel therapeutic approaches during cholinergic dysfunction.

5.2 Presynaptic Response to VACHT

We revealed that in B6eGFPChAT mice, VACHT overexpression occurs independently of significant changes in ChAT, CHT or AChE protein levels, or ChAT and AChE enzymatic
activity. Some studies have shown that these presynaptic components of cholinergic neurons that allow for the synthesis of acetylcholine are able to self-regulate to maintain normal output of acetylcholine to the synaptic cleft (Brandon et al., 2004; Volpicelli-Daley et al., 2003). Our findings, along with a recent study using a novel mouse model of VACHT overexpression (Kolisnyk et al., 2013) suggest that changes in VACHT do not elicit similar changes. In a system with clear mechanisms for auto regulation, it is intriguing that in B6eGFPChAT mice, increased VACHT expression does not decrease CHT or ChAT protein levels, or alternatively, increase AChE expression. Potential mechanisms for the regulation of these proteins, and lack of compensatory regulation in B6eGFPChAT mice, are discussed below.

The first potential trigger for a compensatory response within the cholinergic neuron following VACHT overexpression is the increased acetylcholine content within synaptic vesicles. The ability of some monoamine-containing vesicles to sense their neurotransmitter content has been demonstrated. In these cases, vesicular heterotrimeric G proteins are activated by luminal neurotransmitter content (Brunk et al., 2006; Höltje et al., 2003), which are believed to modulate chloride dependence (Winter et al., 2005) and thereby regulate transport across the vesicular membrane. Acetylcholine transport is not chloride-dependent which suggests that these particular mechanisms do not contribute to acetylcholine loading following VACHT overexpression. Alternative mechanisms for luminal content regulation of vesicular packaging have not been revealed. These findings predict that regulation via vesicular content following increased or decreased VACHT expression is unlikely.

The second potential trigger for compensation would be enhanced quantal release of acetylcholine to the synaptic cleft. Direct evidence suggests that the normal quantal release of acetylcholine does not saturate postsynaptic receptors (Hartzell et al., 1975). The additional synaptic acetylcholine released during VACHT overexpression presumably binds additional postsynaptic receptors and if saturated, additional molecules are either first to be broken down by AChE, or fill in the receptor binding sites after the initial acetylcholine molecules exit. Excess substrate may also be processed by butyrylcholinesterase, an enzyme that hydrolyses acetylcholine along with other esters, and exhibits substrate activation (Mesulam et al., 2002). Considering that AChE protein and activity levels are normal in B6eGFPChAT mice, acetylcholine is expected to be rapidly degraded, even under conditions of VACHT
overexpression. For these reasons, acetylcholine should only be present in the synaptic cleft for fractionally longer than under normal conditions. Preservation of the temporal pattern of acetylcholine release may also reduce the potential for post-synaptic desensitization due to the prolonged overabundance of neurotransmitter, although this remains to be experimentally determined (see Chapter 7).

The third potential for compensation relates to the inevitably high levels of residual extracellular choline that results from increased acetylcholine hydrolysis. Under these conditions, one might expect up-regulation of CHT levels similar to that which occurs in ChAT- and AChE-deficient mice (Brandon et al., 2004; Volpicelli-Daley et al., 2003). Our study of CHT mRNA and protein expression levels did not find this to be the case (Chapter 2). It must be considered that post-translational trafficking of CHT may occur, which would not have been revealed by our immunoblot analysis (Ferguson et al., 2003), however this mechanism of CHT regulation has only been established following activity-dependant presynaptic depolarization (Ferguson et al., 2003). It is unclear whether elevated extracellular choline alone can drive CHT mobility. Consistent with our findings, other studies suggest that high extracellular choline levels are better tolerated by cholinergic neurons than choline depletion (Mellott et al., 2007). Several mechanisms exist to clear acetylcholine-derived choline including the buffering of brain extracellular choline levels by plasma choline levels, concentration dependent choline uptake by low-affinity transporters and CHT (Sarter and Parikh, 2005). Under normal conditions, these mechanisms lead to the rapid clearance of depolarization-induced acetylcholine release in the timeframe of 5-8 seconds (Parikh et al., 2004). The elevated uptake of choline that may result from increased acetylcholine hydrolysis would likely be tolerated by ChAT activity, which is normally present in kinetic excess (Brandon et al., 2004).

Within these considerations, the moderate elevation of VACht protein levels in B6eGFPChAT mice is well positioned to enhance acetylcholine release that, as we support in our study, occurs in the absence of compensatory regulation of acetylcholine synthesis, or choline recycling.
5.3 Peripheral Response to VACHT

5.3.1 Presynaptic Cholinergic Neurons

Peripheral neurons exhibit unique characteristics compared to central neurons (see Chapter 1). Mammalian peripheral nerve terminals are ~300 µm³ in volume and contain tens or even hundreds of thousands of synaptic vesicles (Birks et al., 1960), whereas central terminals are several orders of magnitude smaller and contain only a couple of hundred synaptic vesicles (Denker and Rizzoli, 2010; Rizzoli and Betz, 2005). The compact nature of central synapses allows for enhanced density and increased complexity of central neuronal networks. However, these features also make central terminals vulnerable to depletion of neurotransmitters. In both peripheral and central neurons, two mechanisms are crucial to sustain neuronal activity through prolonged stimulation. First, mechanisms must be in place for released synaptic vesicles to be recycled after their release. Classically, vesicles are thought to fuse to the synaptic membrane and rapidly discharge their contents to the synaptic cleft. Following neurotransmitter release, vesicles are retrieved via clathrin-mediated endocytosis (Heuser et al., 1979), however small fusion pores may also mediate neurotransmitter release and accelerate vesicle recycling (Zhang et al., 2009). Second, vesicles must be rapidly refilled with neurotransmitter to provision their reintegration into the recycling vesicle pool and allow for their subsequent release (Denker and Rizzoli, 2010). In peripheral neurons, their large reserve of neurotransmitter-containing vesicles delays the dependency of fast recycling and refilling of acetylcholine compared to central terminals. However, under certain physiological scenarios in both peripheral and central pathways, such as when synaptic vesicles cycle faster than they can be filled (Searl et al., 1990), neurotransmitter transporters may be rate limiting to neurotransmitter release.

In our study, we did not observe significant peripheral motor effects in B6eGFPChAT mice compared to controls. These results were not surprising, as previous studies support a large buffering capacity against changes in cholinergic tone in peripheral motor neurons (Guzman et al., 2011; Prado et al., 2006). It is worth noting that the assays used to assess peripheral motor function in these studies involve relatively acute bouts of activity that are not likely to deplete the peripheral acetylcholine pool. Some studies have identified hypercholinergic phenotypes through the use of endurance-related behavioural paradigms. An example of this is the rodent treadmill. In these paradigms, mice are trained to run on a treadmill at high speed and incline angle, with
compliance ensured through a mild electrical shock if the animal falls off. Using this approach, motor neuron specific overexpression of CHT (Lund et al., 2010) as well as global overexpression of CHT (Holmstrand et al., 2013) and VACHT (Kolisnyk et al., 2013) led to increased motor performance. As none of these models exhibited changes in grip strength or performance on the wire hang test, the factor assessed by the treadmill does not appear to be impacted by cholinergic signalling capacity or changes in muscle tone per se. Instead, the phenotype is only revealed under conditions that are taxing on cholinergic neurotransmission. Presumably, under these conditions, the acetylcholine-containing vesicular reserves in large peripheral terminals are depleted more readily in control animals versus those with enhanced synthesis or packaging of acetylcholine. Although not measured in this thesis, treadmill performance may also be enhanced in B6eGFPChAT mice, however to what extent remains to be determined (see Chapter 7). Changes in motor endurance are typically evaluated during one hour long exposures on the treadmill (Kolisnyk et al., 2013; Lund et al., 2010) and it is therefore not expected that potential changes in motor endurance in B6eGFPChAT would affect the other acute, short-term procedures used in this thesis.

5.3.2 Metabolism and Homeostasis

In addition to peripheral motor function, we studied the effect of VAChT overexpression on metabolic characteristics as a surrogate to evaluate the effect of enhanced cholinergic input to sympathetic and parasympathetic targets. We evaluated the RER (energy source) and EE (energy used) utilized during a 24-hour period and revealed that no significant differences existed between genotypes. Despite cholinergic signalling being important in the autonomic system, these results agree with the majority of studies suggesting that modified cholinergic function does not significantly impact RER or EE (Androne et al., 2003; Kolisnyk et al., 2013). The exception to this are mice with striatal-specific deficit in acetylcholine release have been shown to have increased RER, suggesting that the control of energy source appears to be regulated, at least in part, by cholinergic signalling. While the mechanism is unclear, sympathetic cholinergic stimulation of the adrenal gland stimulates glycogenolysis through stimulated release of glucose (epinephrine) and inhibited release of insulin (norpinephrine) in the liver, adipose tissue and skeletal muscle (Bray, 1967). Accordingly, VACHT deficient mice exhibit an expected increase in blood insulin levels (Guzman et al., 2012). The lack of observed effect on RER in this thesis,
along with other studies that elevate levels of acetylcholine (Androne et al., 2003; Kolisnyk et al., 2013) may indicate that the tonic activation of sympathetic cholinergic pathways to the adrenal medulla are regulated through some other, non-cholinergic mechanism. Given that the effect of AChE inhibitors on catecholamine release can be experimentally determined during conditions of low cholinergic tone (i.e. aging and AD) (Guzman et al., 2012; Petrie et al., 2001), an assessment in aged B6eGFPChAT mice, with age-dependant decrease in cholinergic function, may be a good candidate for detecting VACHT-mediated changes in RER. Future studies could be directed toward this hypothesis.

5.4 VACHT and Cognition: Novelty Induced Locomotion

The absence of peripheral behavioural effects in 4-month old B6eGFPChAT mice compared to controls allows for an isolated analysis of central cholinergic functions. Of specific interest in this thesis, we wanted to evaluate the effect of VACHT overexpression on central cognitive function. Within the peripheral measures studied, the peripheral phenotypes of B6eGFPChAT and control mice at 4-months of age (baseline for subsequent longitudinal and cross-sectional aging studies) were not significantly different.

One of the simplest systems to induce cognitive processing is the exposure to novel environments. Acetylcholine has been shown to regulate a vast range of responses to novel information. As is set out below, this includes increasing exploratory behaviours during exposure to novel environments, but also includes changes to hippocampal and cortical function including enhanced synaptic plasticity, components of adult neurogenesis and the reduction of proactive interference of novel information through encoding-retrieval scheduling. The latter effect will be discussed in detail in Section 5.6.

5.4.1 Enhanced Response to Novelty

Chapter 3 demonstrates that increased VACHT expression affects locomotor activity. Importantly, these results show that the affect of increased VACHT on locomotion is dependent on the environment, or more specifically, the novelty of that environment. We found that increased VACHT decreases spontaneous locomotion in a familiar environment and increases novelty-induced locomotion upon immediate entry into the OF. Additionally, our results show that increased VACHT impairs locomotor habituation in the OF.
Exposure to a novel environment elicits several processes including exploration, arousal, attention, anxiety and fear (Crawley, 2007). This response is required as the animal becomes acquainted with the environment and is thought to be derived from the animal establishing availability of food and risk from predators. During initial exposures, increased locomotion is thought to reflect the combined emotional and exploratory response, and has been shown to be regulated through both cortical and hippocampal cholinergic activation (Giovannini et al., 2001). Repeated exposure to the same environment attenuates locomotor activation, as behavioural habituation, mediated in part by septohippocampal cholinergic activation, reduces the emotional contribution (Giovannini et al., 2001). Therefore, the locomotor response in subsequent exposures to the environment represents exploratory behaviour (Giovannini et al., 2001). Voluntary exploratory behaviour is often considered an indication of corticostriatal neurotransmission, particularly that mediated by striatal dopaminergic primary neurons. Cholinergic interneurons located within the striatum act to modulate striatal output. Therefore, VACHT overexpression can potentially contribute to the attenuation of locomotion through striatal-modulation, or the enhanced locomotion through augmented cortical and hippocampal activation.

Our finding that VACHT overexpression attenuates locomotor activity in a familiar environment coincides well with enhanced dopaminergic inhibition by cholinergic interneurons in the striatum. On the other hand, our finding that VACHT overexpression augments locomotor activity in a novel environment suggests a role for cortical and hippocampal activation. Many studies have illustrated a strong correlation between hippocampal acetylcholine release and locomotor activity related both to exploration and diurnal variations (Kikuchi et al., 2013; Takase et al., 2014). Unfortunately, our understandings of causal aspects of this relationship are unknown. Our findings, and those of others illustrating enhanced locomotor activity following augmentation of cholinergic function (Holmstrand et al., 2013), suggest that cholinergic activity may specifically contribute to enhanced novelty-induced locomotion.

In an attempt to further elucidate the role of VACHT overexpression on novelty-induced locomotion, we performed a habituation study where the animals were re-exposed to the OF at two additional time points. The hypothesis was that as the animal habituated, the contribution of cortical and hippocampal contribution would decrease, and a hypoactive phenotype would be
revealed. In contrast, our findings revealed that VACHT overexpression impaired typical patterns of exploratory habituation. Two possibilities can explain this phenomenon. First, habituation is considered one of the most elemental forms of memory, and as such, it is tempting to conclude that VACHT overexpression impairs associative learning. In our model however, we did not detect impaired learning in other paradigms (see Chapter 4). Second, impaired habituation (measured through locomotor activity) may be the result of the short-term locomotor hyperactivity upon entry to the novel environment, presumed to be related to the instantaneous release of acetylcholine (Giovannini et al., 2001; Thiel et al., 1998). Indeed, post-hoc analysis of distance accumulated over time during the habituation trials revealed significant differences between genotypes within only the first five minutes of exposure, after which point there were no differences in locomotor activity (data not shown). Taken together, this suggests that the hyperactive response in VACHT overexpressing mice is highly dependent on the first five minutes of exposure. Accordingly, this 5-minute window is when novelty-induced acetylcholine release, and in turn, augmentation by VACHT overexpression, is greatest (Giovannini et al., 2001; Thiel et al., 1998).

5.4.2 Disinhibition of Anxiety

In Chapter 3, our results suggest that increased VACHT expression decreases anxiety-like behaviour. However, it is currently not clear whether the observed change in anxiety-like behaviour is related to direct modulation of emotionality or if it is related to the observed change in novelty-induced locomotion.

Recent studies have not clarified this issue. In CHT-BAC mice, anxiety-like behaviour is increased, in contrast to our observations (Holmstrand et al., 2013). In the same study, CHT-BAC mice were also found to be hyperactive in the novel OF, similar to our observations. In ChR2-ChAT-EYFP mice, no change in anxiety-like behaviour (or locomotor activity) was observed (Kolisnyk et al., 2013). These findings are difficult to reconcile, as they are part of a number of mixed results regarding the influence of cholinergic neurons on anxiety. For example, nicotine at high dose in the hippocampus is anxiogenic whereas at low dose, it is anxiolytic (File et al., 2000b). Further complicating these interpretations is the observation that the cholinergic contribution to anxiety appears dependent on the level of stress generated by the test itself. For example, the anxiogenic effects of nicotine are induced only in brightly lit novel environments.
(File et al., 1998; File et al., 2000a). These results predict that the influence of cholinergic tone on anxiety-like behaviour is dependent on the level of stress within the subject.

In our study, it is difficult to eliminate the contribution of increased novelty-induced locomotion on some outcome measures of anxiety-like behaviour. Specifically, the number of transitions between fields is particularly susceptible to deviations in overall activity. Proportional measurements on the other hand (i.e. the time spent in the open arm of the EPM), are relatively insulated by changes in locomotor activity, and with this in mind, an argument could be made to suggest that increased VACHT expression is anxiolytic. This is supported by findings that cholinergic agonists directly injected in the hippocampus decrease EPM anxiety (Bannerman et al., 2004; Degroot and Treit, 2002).

A role for the hippocampus in the mediation of emotionality has gained traction within recent years. For example, lesions of the ventral hippocampus specifically reduced anxiety in unconditioned tests including the EPM. Conversely, activation of the ventral DG results in significant decreases in EPM anxiety, possibly mediated through excitatory afferents between the hippocampus and amygdala (Kheirbek et al., 2013). The precise role for the ventral hippocampus in anxiety is largely unknown, however it may recognize conflict or uncertainty by detecting mismatch between the current state and memory (Bannerman et al., 2014).

Some studies have linked dendritic plasticity within the DG with anxiety-like behaviour (Leuner and Shors, 2013). In general, chronic stress leads to dendritic retraction and reduced branching of neurons in the hippocampus (Watanabe et al., 1992). Emerging evidence now predicts the reciprocal relationship between newborn dendritic complexity and anxiety-like behaviour. Specifically, mice lacking functional brain derived neurotrophic factor (BDNF) receptors exhibit poor dendritic arborisation of newborn neurons and anxiogenic performance in the EPM (Bergami et al., 2008). Similarly, mice expressing a BDNF variant also exhibit anxiety-like behaviour that is correlated with impaired dendritic arborisation (Chen et al., 2006). An exciting finding of this thesis suggests that enhanced cholinergic signalling may foster an environment permissive for newborn dendritic expansion in the DG (see Chapter 4). These findings, subsequent to our initial report of anxiety-like behaviour in B6eGFPChAT mice, supports the hypothesis that structural changes of hippocampal dendrites may themselves contribute to the anxiolytic phenotype observed in the EPM.
5.5 VAChT and Cognition: Hippocampal Structure and Function

Acetylcholine has been associated with changes in hippocampal structure and function including enhanced synaptic plasticity, components of adult neurogenesis and the reduction of proactive interference of novel information through encoding-retrieval scheduling. In the case of enhanced synaptic plasticity, acetylcholine has been implicated in the induction of long-term potentiation (LTP), a long-lasting enhancement of synaptic transmission (Gu and Yakel, 2011; Kanju et al., 2012; Martyn et al., 2012). Specifically, in mice with forebrain VAChT deficiency, LTP is impaired while long-term depression, a long-lasting enhancement of synaptic transmission is spared (Martyn et al., 2012). The effect of VAChT overexpression on LTP was not evaluated in this thesis, or in related studies (Kolisnyk et al., 2013). Given the previous findings mentioned above, B6eGFPChAT mice are candidates for enhanced LTP, however this will require subsequent study. In our model, we observed dendritic expansion in newborn neurons, and the implication of these findings in the context of cognition will be discussed.

5.5.1 Ramification of Newborn Dendrites

Our results presented in Chapter 4 indicate that increased VAChT expression increases the dendritic complexity of newborn neurons in the DG. This finding marks the first report that cholinergic innervation specifically enhances the dendritic expansion of newborn neurons and highlights the important role for cholinergic innervation to the hippocampus during the maturation of immature neurons during adult neurogenesis.

It is well established that newborn neurons integrate into the existing neuronal network. Approximately 7-day old immature neurons extended dendritic processes to towards the molecular layer of the DG, where their dendrites ramify. By approximately four weeks, newborn neurons show polarized morphology similar to the existing dentate neurons (Zhao et al., 2008). The period from 1-4 weeks is marked by DCX expression, a microtubule associated protein that is believed to enhance microtubule polymerization during dendritic growth (Gleeson et al., 1999). GABA-mediated depolarization, Disc1 activity, and neurotrophic factors, such as BDNF and their downstream signals, have been implicated in neurite extension of newborn granule cells (Chen et al., 2006; Duan et al., 2007; Ge et al., 2007). Our results indicate that VAChT-mediated enhancement in acetylcholine can also contribute to dendritic ramification. Acetylcholine has
been shown to play a yet undetermined role in the dendritic expansion of cortical pyramidal cells during early development (Hohmann et al., 1991). It seems plausible that there are conserved mechanisms for dendritic maturation during early development and the integration of newborn neurons in the adult brain (Laplagne et al., 2006). Given previous findings, acetylcholine may be related to some or all of the identified mechanisms for enhanced dendritic outgrowth mentioned above. First, acetylcholine regulates the maturation of GABA-mediated depolarization. Second, increased acetylcholine through the septohippocampal pathway elevates levels of neurotrophic factors, specifically BDNF (Knipper et al., 1994; Lapchak et al., 1993). Furthermore, Disc1 expression may be mediated by the neuregulin family of proteins through nAChRs (Hancock et al., 2008). However, neuregulin deficient animals do not show early structural changes in dendritic processes, only effects on dendritic spines (Barros et al., 2009), suggesting that the morphological consequence of Disc1 deletion may be independent of neuregulin dependant nAChR activity.

While the evidence of the causal effect of acetylcholine on dendritic ramification during both early development and the integration of newborn neurons are limited, the new findings set out in this thesis offer support for the involvement of acetylcholine. Furthermore, the implication of hippocampal dendrites in emotional and cognitive function warrants further studies to elucidate the mechanism for VAChT-induced dendritic expansion.

5.6 VAChT and Cognition: Implications of VAChT in memory systems

In B6eGFPChAT mice, we observed enhanced novelty-induced locomotion, a reduction of anxiety-like behaviour in novel environments, and enhanced dendritic ramification in newborn hippocampal neurons. The specific functional consequences of these changes towards memory are discussed below.

The finding of enhanced novelty-induced locomotion positively affects the level of exploration in an environment. Clearly, encoding a new environmental context and its contents, or refining a previous memory trace, is more efficient when the environment is sampled from multiple viewpoints. As supported in our model, both locomotor activity and the number of rearings are increased during a novel exposure (Giovannini et al., 2001) and this activity is strongly
modulated by hippocampal acetylcholine (Giovannini et al., 2001; Thiel et al., 1998). In addition to physical sampling of an environment, high hippocampal acetylcholine has been proposed to prioritize encoding activity patterns within the trisynaptic circuit. In this system, acetylcholine inhibits recurrent (CA3-CA3) and Schaffer (CA3-CA1) collaterals with minimal effect on EC-CA1 input via the perforant path (Easton et al., 2012). Simply put, acetylcholine protects to-be-encoded input patterns from the proactive interference arising from memory retrieval systems.

Our second finding of enhanced dendritic expansion within the newborn neurons of the DG may be implicated in the encoding-retrieval scheduling in the hippocampus. Increasing the surface area of dendrites in granule cells increases the substrate for excitatory input to the DG. In addition, longer dendritic branches may concentrate distal connectivity and enhance the excitatory input on hippocampal neurons (Müller et al., 2012). As such, under these conditions, DG-originated mossy fiber input to the CA3 may be selectively enhanced. Interestingly, separate lesion of the DG and the perforant path to the CA3 have revealed a double dissociation between these closely associated paths, with EC-DG signalling specifically supportive of encoding (Lee and Kesner, 2004). As such, the enhanced dendritic expansion in B6eGFPChAT mice may possess the capacity to enhance memory encoding.

5.6.1 Enhanced Spatial Resolution

The combination of enhanced novelty-induced exploration, with increased dendritic complexity within the newborn neurons of the B6eGFPChAT hippocampus is congruent with a hypothesis of enhanced cognitive performance. Our findings in Chapter 4 indicate that increased VACHt expression does in fact improve spatial resolution in aged mice longitudinally tested in the MWM.

During the MWM acquisition trials, 24-month old VACHt overexpressing mice used successively shorter escape distances than controls. During the final trial of the exposure, VACHt mice were significantly better at locating the hidden platform than controls. Of interest, while the escape distances deviated between genotypes, the escape latency was the same. This lead to a change in the swim speed which was attributed to the change in distance but not latency. The fact that mice utilized a shorter route led us to hypothesize that VACHt overexpression may improve path efficacy.
Previous studies using mice with elevated adult neurogenesis have revealed improvement in spatial precision without corresponding changes in escape latency (Garthe et al., 2009; Gil-Mohapel et al., 2013). Applying a similar analytical approach, our results show that VACHT overexpression improves the spatial precision of the search as mice tracked a more direct approach to the hidden platform location.

Although improvement in spatial acquisition was observed, there were no clear differences in spatial memory. Some studies predict age-related changes in spatial memory (Bellush et al., 1996) while others do not (Kennard et al., 2013). One potential explanation for the lack of a spatial memory effect would be that that even though VACHT overexpressing mice completed the acquisition session with superior performance, each genotype demonstrated that they had learned the task. In other words, the contribution of acquisition training may had met asymptote with regards to its contribution on spatial memory. To test this, the probe trial could have been made more challenging if it were given at an earlier point. Probe trials incorporated into the acquisition period are common in MWM research (Magnusson et al., 2003). To explore this post hoc, we considered escape latency and distance for Trial 1 of Day 2 (data not shown) which represents the first attempt to locate the platform after a 24-hour retention interval. No significant differences were found during this trial, and it therefore does not seem likely that a traditional probe trial conducted earlier in training would have yielded different results.

5.6.2 Longitudinal Spatial Memory Design

Cross-sectional studies define cohorts of subjects that share a particular event, often their date of birth. Cohorts can then be evaluated at, or around the same time, quickly yielding results that can be generalized to other populations with similar characteristics as the study cohorts. Age-dependant changes in rodent cognitive function are typically evaluated using cross-sectional study designs. It is however, difficult to interpret outcomes of cross-sectional studies as related to aging per se, and not to other cohort effects on normal development. For example, a 6-month old rodent may have been exposed to different environmental factors (i.e. seasonal variation, maternal handling, etc.) during the first months of life compared to a 12-month old cohort. Longitudinal studies, where single cohorts are repeated tested over time, are better suited for studying the effect of aging within a given cohort, as they control for external factors affecting development. The benefit of this approach must be considered against experimental practicality,
as some studies require the sacrifice of the subject at defined time points, precluding longitudinal assessment.

Numerous studies have evaluated spatial cognition using the MWM in aged rodent cohorts (see Kennard and Woodruff-Pak, 2011 for review), however few have utilized a longitudinal design (Vicens et al., 1999; Vicens et al., 2002; Wong and Brown, 2007). The results of cross-sectional versus longitudinal studies are unique and likely reflect how each design addresses a unique research question; how does a treatment affect a naive cohort of animals at different ages versus how does a treatment affect the same animals during their aging process. Unlike cross-sectional studies that have typically demonstrated decreased MWM performance in aged cohorts, Wong and Brown observed that B6 mice exhibited equal or superior spatial acquisition in aged mice, with no associated change in spatial memory. The preserved performance in longitudinal designed MWM studies can be explained by the work of Vicens and colleagues (2002) who revealed that previous training augments future MWM performance. The ability of previous training to a task to improve subsequent exposures has been called the longitudinal “learning effect”. The learning effect appears to last for approximately 8-10 months, after which point the previous exposure has little effect on subsequent MWM performance.

In our study, B6 mice exhibited a slight decline in probe performance between 4 and 18 months, while performance was restored by 24-months of age. This phenomena may reflect the previously observed decay of the learning effect (Vicens et al., 1999; Vicens et al., 2002). In contrast, B6eGFPChAT probe performance was maintained throughout aging. It is possible that enhanced hippocampal plasticity in B6eGFPChAT mice contributes to superior long-term memory and reactivation of the earlier training, beyond the typical duration of the learning effect.
Chapter 6
Thesis Considerations

6.1 Use of the Morris Water Maze in Overexpression Studies

The studies detailed in this thesis utilize the B6eGFPChAT mouse model which presumably exhibits global overexpression of VAChT. Confirmation of increased protein expression was conducted in the hippocampus, striatum, cortex, heart and small intestine. Confirmation of elevated acetylcholine release was confirmed in ex vivo hippocampal slices. Cholinergic innervation influences a myriad of targets, and although we utilized behavioural outcomes with defined anatomical-dependence in the literature, it is impossible to exclude the influence of other cholinergic paths. With the findings of this thesis in mind, it may be worth evaluating models with site-specific overexpression of VAChT to further isolate the contribution of hippocampal acetylcholine with the observed phenotypes in this thesis (see Chapter 7).

In addition, a limitation of the MWM is that observed differences between aged mice may be an artifact of the spatial memory task itself. Specifically, at younger ages (4- and 18- months) we are seeking enhanced memory performance above non-impaired levels. The MWM was created and has been primarily validated using models of impaired memory function (Morris, 1984), and as such it may not be sensitive or specific enough to detect greater than “normal” levels of performance. In aged mice (24-month old) normal cholinergic decline generates a system where we may be detecting impaired performance (in B6 mice) versus normal performance (in B6eGFPChAT mice).

While this technical limitation is not expected to diminish the findings of improved performance in 24-month B6eGFPChAT mice, it may have reduced the ability to detect improved performance in younger mice. In these cases, special approaches to the MWM could have been of benefit, and rely on the increased hippocampal-dependency of the task. For instance, it has become more common to include a spatial reversal (opposite quadrant) or shift (adjacent quadrant) phase following the MWM probe trial (Crawley, 2007). Reversal learning in the MWM reveals whether or not animals can extinguish their initial learning of the platform’s position and acquire a direct path to the new goal position. The hippocampal flexibility required to successfully perform this task may reveal differences in young B6eGFPChAT mice.
Particularly because spatial precision during the acquisition trial appears to be modulated by cholinergic tone, a spatial reversal or shift with a small platform may unmask subtle improvements in B6eGFPChAT mice. Future studies utilizing these designs may be essential to further characterize the effect of increased VAChT expression.

6.2 Homozygous Comparison of BAC-derived Transgenic Mice

Mice with targeted transgenes are usually derived from hybrids of two mouse strains. Most commonly, gene targeting is carried out in embryonic stem (ES) cells derived from the mouse strain 129 (Gerlai, 1996). 129-type ES cells carrying the transgene are introduced to blastocyst stage embryos generating chimeric mice which are then mated to B6 mice. The mixed genetic background in the segregating population complicates genetically-driven phenotypes in these mice. For example, recombination patterns (containing both 129 and B6 alleles) may be different between littermates, suggesting that even non-transgenic mice from a population may not accurately represent a counterpart to transgenic mice (Crusio et al., 2009; Gerlai, 1996). This limitation can be alleviated through large sample sizes to decrease the sampling error associated with recombination. However, the complication of the flanking allele problem is not as readily solved (Gerlai, 1996). Because the probability of recombination is inversely proportional to the distance between loci of the genes, 129-derived genes flanking the transgene are likely to be maintained in transgenic mice. Conversely, non-transgenic mice will have a low probability of these 129-derived alleles. Consequently, phenotypic differences may be due to the transgene or background genes associated with the targeted locus.

In contrast to ES-derived transgenic mice, the B6eGFPChAT mice used in this thesis were generated through direct microinjection of BAC transgenes into the pronucleus of fertilized oocytes (van Keuren et al., 2009). In this scheme, gene targeting is performed in B6-derived BACs and delivered to B6 recipients, thus eliminating both heterologous recombination patterns and flanking alleles. In addition, BAC-derived transgenic mice more frequently represent the expected expression patterns, likely due to reduced influence of position effects insulated by the sheer size of BAC transgenes (Giraldo and Montoliu, 2001). The large capacity of BAC vectors can also be of benefit when the exact promoter region is unknown, allowing for large portions of 5’- and 3’-flanking sequence. In B6eGFPChAT mice, approximately 78kb and 36kb of DNA
flanked the upstream and downstream ends of the ChAT locus, capturing the entire known promoter region of the cholinergic gene locus (Eiden, 1998).

While the BAC transgenic approach eliminates a number of phenotypic uncertainties that exist with ES-derived models, the insertion of large fragments of DNA may interrupt one or more endogenous transcripts. Integration of BAC transgenes occurs at random insertion sites in the genome (van Keuren et al., 2009), however trends in insertion studies predict that there may be regions selected more often for integration than others (Hamada et al., 1993; Rijkers et al., 1994). Confirming these patterns in B6eGFPChAT mice was not trivial. In B6eGFPChAT mice, circularized (and not linear) BACs were injected, making primer design for the identification of the BAC-genome interface challenging as the site of linearization was unknown. Nevertheless, an undertaking to identify the insertion site was performed by predicting sites of linearization and designing primers for inverse polymerase chain reaction (data not shown). Unfortunately, this project proved unsuccessful, and to this date, the insert location of the BAC transgene in the B6eGFPChAT model and other ChAT-spanning BAC models (Heiman et al., 2008; Kolisnyk et al., 2013) are unknown. Alternative approaches to resolve the site of insertion, and any potential interrupted gene products, could be performed using other chromosome walking techniques, or ultimately whole genome sequencing.

In our study, homologous B6eGFPChAT mice and congenic B6 controls were directly compared. This comparison is made possible given that, as stated above, background genes are absent (Crusio et al., 2009; Gerlai, 1996). However, comparing two pools of mice from different maternal units may introduce indirect environmental effects of the transgene (Crusio et al., 2009). Specifically, we cannot eliminate the potential that maternal care may be affected in B6eGFPChAT and that this developmental deviation could contribute to the phenotypes observed in this thesis. While heterozygous mating would generate littermates under the same maternal care, thereby alleviating this confound, the relatively low copy number, and marginal overexpression of VACht in the B6eGFPChAT model likely precludes this approach. To support our findings, and alleviate phenotypic differences related to environment between the B6eGFPChAT model and the controls, we performed comparison to the published literature related to VACht depletion, drawing ‘rescue-like’ contrasts to our findings where possible (Gerlai, 1996).
Chapter 7
Future Directions

The work presented in this thesis opens new areas of research linking the fields of cholinergic neurotransmission, neuronal plasticity including neurogenesis, cognitive functions and aging. Several additional experiments directly stem from these projects and have been explained throughout Chapters 2-6. As such, they are not mentioned below. Instead, the following sections will focus on new research directions and applications that can be considered in the context of this thesis.

7.1 Localized Elevation of Acetylcholine Release in the Hippocampus

A central limitation to the work conducted in this thesis relates to the use of a congenic mouse model that exhibits global overexpression of VAChT. Although we utilized behavioural outcomes with well-defined anatomical dependence in the literature, it is impossible to exclude the influence of other cholinergic paths. Localized overexpression of VAChT in the hippocampal terminals would offer the ability to specifically evaluate the effect of enhanced acetylcholine release in the hippocampus. Cre-lox recombination has been widely utilized to carry out site-specific deletion, mutation and insertion of transgenes (Orban et al., 1992). This well characterized approach can be exploited to enhance the site-specificity of VAChT overexpression.

To achieve this, a slight modification of the RP23-268L19-EGFP transgene used to generate the B6eGFPChAT mice will be required. Using a specialized shuttle vector for recombination with BAC transgenes (Lee et al., 2001), a modification cassette consisting of the mCherry coding sequence followed by a termination signal, flanked by loxP sites, will be generated. Homologous arms flanking the stop cassette will be used allowing for insertion of the stop cassette directly upstream of the VAChT coding region. The resulting transgene would be identical to that present in B6eGFPChAT mice, with the exception that transcription is terminated following mCherry expression, and before VAChT expression. Modified RP23-268L19-EGFP BACs would be then selected and microinjected into fertilized embryos using pronuclear injection, as was performed for the creation of B6eGFPChAT mice (Tallini et al., 2006), resulting in mice with normal
VAChT expression. These modified mice, containing a floxed mCherry sequence, would then be crossed to the Cre-recombinase mice to target site-specific removal of the stop cassette, thus driving VAChT overexpression. Transgenic expression of mCherry and GFP would indicate transmission of the transgene (mCherry expression) and the presence of Cre recombination (GFP expression).

A candidate for hippocampal-specific VAChT overexpression using the proposed model utilizes the commercially available disks large homolog 3 (DLG3)-Cre mouse (Tg(Dlg3-cre)KG118Gsat/Mmucd). DLG3 is a synaptic scaffold protein specifically expressed within the CA regions of the hippocampus and the DG. Importantly, dlg3 is present on cholinergic neurons where it is thought to provide scaffold for nAChRs (Conroy et al., 2003). Genetic cross of DLG3-Cre mice with our proposed model would potentially lead to hippocampal overexpression of VAChT. While this modification would not enhance cholinergic input originating from the basal forebrain cholinergic neurons, it would elevated acetylcholine release from cholinergic interneurons within the hippocampus (Frotscher et al., 2000). The localized release of acetylcholine from these interneurons may be sufficient to stimulate the local post-synaptic AChRs on newborn granule cells, and allow for the evaluation of dendritic ramification and changes in spatial memory related to increased VAChT-mediated cholinergic tone in the hippocampus.

A commercially available model exhibiting Cre-recombinase activity solely in the medial septum does not appear to be available. Nonetheless, potential models do exist that exhibit Cre expression in the basal forebrain, along with expression which typically includes the neocortex, thalamus and brain stem. The Six3-Cre mouse is of particular interest, considering it has been shown to drive Cre expression in cholinergic neurons of the ventral forebrain (Furuta et al., 2000). VAChT-deficient mice exploiting the spatial expression of the Six3-promotor have deficiency in acquisition of hippocampal-dependant spatial tasks and hippocampal LTP, and are hyperactive, likely as a result of decreased cholinergic tone in the basal ganglia (Martyn et al., 2012). Given this previous study, the Six3-Cre model may offer increased regional specificity to associate the observed cognitive and locomotor behaviour in this thesis with VAChT overexpression.
Using the DLG3-Cre or Six3-Cre mice to drive expression along the septohippocampal pathway, as an example, it would be of value to repeat the assessment of dendritic ramification in hippocampal newborn neurons and the longitudinal assessment of spatial memory in the MWM during aging. Ultimately, the use of these models could further clarify the site-specific contribution of VAChT overexpression on the phenotypes observed in this thesis.

7.2 Application to Pathological Conditions: Alzheimer’s Disease

In addition to normal aging, patients with AD have amyloid-beta peptide (Aβ) pathology which can exacerbate cholinergic dysfunction and cognitive impairments (Francis et al., 1993; Perry et al., 1978; Sims et al., 1983; Wilcock et al., 1982). These clinical observations, supported by the observation that the cholinergic agonist physostigmine reverses scopolamine induced memory impairments in preclinical models, led to the cholinergic hypothesis of memory dysfunction to explain AD pathology (Bartus et al., 1982; Bartus, 2000). While cholinergic decline is not predicted to be the cause of AD, it is a pathological feature sensitive to therapeutic intervention. The clinical application of AChE inhibitors is one of only two classes of approved therapeutics indicated for AD patients, the other being a glutamatergic antagonist.

Acetylcholinesterase inhibitors are currently used in AD patients to prevent the breakdown of acetylcholine at the synaptic cleft, but these approaches do not always result in significant cognitive improvement (Campbell et al., 2008; Lanctôt et al., 2003), possibly because the amount of acetylcholine produced and released has become insufficient to sustain cholinergic neurotransmission. In addition, the extended lifetime of acetylcholine in the synaptic cleft during AChE inhibition may contribute to receptor desensitization. This is particularly relevant for α7-nAChR in the hippocampus that rapidly desensitize (Quick and Lester, 2002). As an alternative approach, strategies focused on increasing the amount of acetylcholine released in the brain during pathological aging, specifically along the septohippocampal pathway, may provide a valuable therapeutic alternative.

VAChT represents a therapeutic target for cholinergic decline during AD, however it has not received much attention aside from the development of diagnostic agents for imaging (Kilbourn, 2013). These radioligands suggest that VAChT density is decreased in AD subjects (Bell and Cuello, 2006; Efange et al., 1997). Furthermore, immunohistochemical studies in postmortem
prefrontal cortical tissue of AD patients supports the loss of VChT immunoreactivity compared to non-AD controls, and was maintained even after correction for decreased levels of synaptophysin in AD tissue (Chen et al., 2011a). These findings predict that the loss of vesicular function in cholinergic neurons may precede the loss of synaptic structure. Increasing VChT expression may be of benefit for the existing synaptic network, despite its degeneration during AD pathology.

Cholinergic therapy has the potential to improve cognition, even when cognitive impairment results from several neuropathological events and deficits in other neurotransmitter systems. To evaluate this hypothesis, I would deliver the ChAT-spanning BAC transgene by crossing B6eGFPChAT mice to a transgenic mouse model of AD. As an example, TgCRND8 mice could be used. These mice overexpress a double mutant form of the human amyloid precursor protein leading to Aβ production. TgCRND8 mice exhibit decreased extracellular acetylcholine in the cortex by 7 months of age with corresponding decrease in ChAT immunoreactivity of cortically projecting cholinergic neurons (Bellucci et al., 2006). In this approach, the global increase in VACChT expression would be of benefit, given that cholinergic depletion appears to be ubiquitous in AD. The resulting hybrid mouse would then be compared to both the B6eGFPChAT and TgCRND8 as controls, to determine the age-related profile of cholinergic decline. In addition to cholinergic tone, the status of cognition could be evaluated in these models. TgCRND8 mice exhibit deficits in MWM spatial memory by 6 months of age, therefore assessment at this discrete time point, or across this time point during an aging study, could establish a therapeutic window. Taken together, these experiments could determine whether VACChT overexpression can protect against Aβ-related cholinergic decline in a mouse model of AD.

7.3 Concluding Statements

In conclusion, the studies completed thus far indicate that the VACChT overexpression in B6eGFPChAT mice increases central acetylcholine release, modulates the dendritic morphology of newborn hippocampal neurons and influences behaviour including locomotion and spatial memory. These data provide partial support of the current hypothesis that enhanced VACChT expression leads to enhanced central and peripheral cholinergic tone and improves hippocampal-dependant spatial memory performance and neurogenesis during aging.
It is important to note that although we have highlighted potential mechanisms by which VAChT-mediated changes in dendritic morphology and behaviour occur, a more thorough examination of these interpretations is required. This is largely due to the global distribution of VAChT overexpression in the current B6eGFPChAT model. The proposed future work offers a foundation to evaluate site-specific overexpression of VAChT, including multiple approaches to specifically elevate acetylcholine in the hippocampus. In addition, as set out above, the future work explores the effectiveness of VAChT overexpression in pathological cases of cholinergic decline, and evaluates the unique potential for VAChT overexpression to increase acetylcholine in the synaptic cleft under endogenous spatial and temporal regulation.

The current results and proposed future directions of this thesis highlight the need to explore strategies to elevate VAChT expression *in vivo*. These therapies may exhibit therapeutic potential for cholinergic dysfunction during aging, in particular for AD.
Chapter 8
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Miniaturized electrochemical system for cholinesterase inhibitor detection

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HIGHLIGHTS

- Acetylcholinesterase inhibitor activity was measured on label-free electrodes.
- Electrochemical detection was validated against Ellman’s colorimetric assay.
- FDA approved acetylcholinesterase inhibitor was implemented as a positive control.
- The IC50 values determined for Donepezil were comparable between assays.
- IC50 were determined using cholinesterase extracted homogenates of C57Bl/6J mice.

GRAPHICAL ABSTRACT


ABSTRACT

The utility of a simple, low-cost detection platform for label-free electrochemical characterization of acetylcholinesterase (AChE) inhibition is demonstrated as a potential tool for screening of small-molecule therapeutic agents for Alzheimer’s disease (AD). Technique validation was performed against the standard Ellman’s colorimetric assay using the clinically established cholinesterase inhibitor (ChEi), Donepezil (Aricept®). Electrochemical measurements were obtained by differential pulse voltammetry (DPV) performed using a portable potentiostat system for detection of the enzymatic product, thiocholine (TCh), by direct oxidation on unmodified gold screen-printed electrodes. The IC50 profile for Donepezil measured in vitro were found to be comparable between both colorimetric and electrochemical detection methods for the analysis of purified human erythrocyte-derived AChE (28 ± 7 nM by DPV; 26 ± 8 nM by Ellman’s method). The selectivity of this unmodified electrode system was compared to a range of biological sulfur-containing compounds including cystine, homocysteine, glutathione and methionine as well as ascorbic acid. Preliminary studies also demonstrated the potential applicability of this electrochemical technique for the analysis of Donepezil in crude cholinesterase samples from anterior cortex homogenates of C57Bl/6J mice.

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1. Introduction

Alzheimer's disease (AD) is the most prevalent form of age-related dementia marked by impairments in cognition, language, and memory [1,2]. Although strong evidence has suggested that processes such as beta amyloid disorganization and tau hyperphosphorylation operate synergistically as key pathogenic factors in disease progression [3-5], available pharmacological treatments have targeted more distal pathways of neurodegeneration. Notably, currently approved AD therapies are limited to two classes of compounds: N-methyl-D-aspartate receptor antagonists and cholinesterase inhibitors (ChEIs) [6,7]. The primary function of these drug therapies is not to impede the proposed antipathologies, but rather to ameliorate behavioral and cognitive dysfunctions, which have significantly delayed - and in some cases avoided - the need for institutionalization [8,9].

In particular, cognitive dysfunctions have been strongly associated with deterioration of cholinergic neurons of the basal forebrain and neocortex, leading to profound deficits in production of the neurotransmitter, acetylcholine [10]. Accordingly, ChEIs have been applied effectively to restore these pathologically reduced neurotransmitter levels by inhibiting native degradation catalyzed by the enzyme acetylcholinesterase (AChE) [11,12], although a range of highly effective ChEIs have been synthesized and well-characterized in vitro, widespread development of novel compounds for clinical testing has been hindered by complications such as insufficient bioavailabilities or adverse drug reactions [13,14]. As a result, only four ChEIs have been approved by the United States Federal Drug Administration for clinical treatment of mild to severe stage AD. These include: Galantamine (REMINYL®), Rivastigmine (EXELON®), Tacrine (COGNEK®) and Donepezil (ARICEPT®) [15]. Though the use of Tacrine has been heavily prohibited in recent years due to high risk for hepatotoxicity [16]. In view of the limited number of available AD therapies, the rapid identification of novel, more biologically compatible ChEIs is necessary.

Ellman's method is currently the standard colorimetric assay for kinetic analysis of AChE activity in vitro, whereby the substrate, acetylthiocholine iodide (ATChI), is enzymatically hydrolyzed in the presence of a thiol-reactive chromogenic dye, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) [17,18]. However, the implementation of extrinsic indicators for determination of enzyme activity poses a number of limitations. For instance, DTNB functions optimally within a specific pH range (pH 6.5-8.5), beyond which chemical decomposition occurs. The presence of other free thiol-containing agents (e.g. glutathione, cysteine) [19,20] or other reducing groups (e.g. oximes) [21,22] present within biological media also cause spontaneous activation of DTNB resulting in inaccuracies during the analysis of crude samples [23-25].

Herein, the limitations of the extrinsic colorimetric reagent, DTNB, were addressed through the implementation of a low-cost electrochemical platform for label-free characterization of AChE activity by direct detection of the enzymatic product, thiocholine (TCh). We have previously demonstrated the oxidation of TCh on unmodified gold electrodes for highly sensitive detection of organophosphate pesticides [26]. In this study, direct TCh detection was employed in combination with a mini-potentiostat and disposable screen-printed electrodes for efficient analysis of reversible ChE activity. Donepezil was synthesized [27] for use as a model ChEI, for which the inhibition activity was characterized with both colorimetric and electrochemical detection systems.

2. Experimental

2.1. Materials and instrumentation

Human erythrocyte-derived acetylcholinesterase (AChE), acetylthiocholine iodide (ATChI, >98%) and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, >98%) were purchased from Sigma-Aldrich (Oakville, ON, Canada). All samples and buffers were of analytical grade, prepared from ultrapure 18.2 MΩ water.

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Fig. 1. Workflow for optical and electrochemical analysis AChE activity: (a) TCh is produced from the enzyme-catalyzed hydrolysis of ATChI and reacts with a chromogenic substrate, DTNB. AChE activity is derived from the formation rate of the optically active product by the change in absorbance measured at 410 nm over time. (b) TCh is detected by direct oxidation on a gold screen-printed electrode. AChE activity is derived from the peak current intensity from TCh oxidation measured at approximately 160 mV vs Ag/AgCl. (c) Images of the miniaturized potentiostat and gold screen-printed electrode system.
obtained from a Cascada LS water purification system (Pall Co., NY, USA). Colorimetric assays were analyzed using a Synergy HT Multimode Microplate Reader (BioTek, Winooski, USA) to measure changes in optical density at 410 nm for a 96-well microtiter plate. All electrochemical measurements were performed using differential pulse voltammetry (DPV) by means of a compact (5.3 x 7.5 x 2.0 cm, 65 g) BDT Ministat 100 potentiostat system obtained from Bio Device Technology Ltd (Kazama, Japan). The low-cost device was used in conjunction with gold screen-printed electrodes (Bio Device Technology Ltd), comprising of a gold working electrode (geometric area: 2.64 mm²), a carbon counter-electrode, and an Ag/AgCl reference electrode (Fig. 1).

2.2. Colorimetric determination of AChE activity

AChE activity was measured using the standard Ellman's colorimetric assay [17] with detailed experimental procedures adapted from more recent studies conducted by Recanatini and co-workers [28–30]. Pilot studies were initially performed to determine the appropriate concentration of AChE to be implemented in Ellman's assay. The optimized reaction velocity of the uninhibited AChE control was set to a rate of 0.100 Δ absorbance at 410 nm min⁻¹ ± 0.030 corresponding to a concentration of 0.1875 μM⁻¹, whereby one unit (U) of human erythrocyte AChE hydrolyzes 1.0 mmole of ATP/Δ min⁻¹ at pH 7.4 at 37 °C. Using a 96-well microtiter plate, purified human erythrocyte-derived AChE (0.1875 U/mL⁻¹) and DNTB (340 μM) were prepared with 50 mM PBS, 100 mM NaCl, pH 7.4 and equilibrated at 37 °C for 10 min either in the presence or absence of inhibitor. Immediately following the addition of substrate, AChE (550 μM), the optical density of each well was simultaneously recorded at 410 nm in 15 s intervals over a period of 240 s. The initial reaction velocity (Δ absorbance at 410 nm min⁻¹) was derived from a linear fit of the absorbance vs. time curves. Percent enzyme activity was determined by comparison of the reaction velocity for the uninhibited control (V₀) relative to inhibitor-treated samples (Vᵢ) by Eq. S1. ChE, Donepezil, was used for cross-comparison of enzyme inhibition screening between optical and electrochemical detection platforms. Synthesis of Donepezil is described in Supplementary Data.

2.3. Electrochemical determination of AChE activity

Sample wells were prepared as described for Ellman's method (see above), save for DNTB, which was substituted with an equivalent volume of phosphate buffer (50 mM PBS, 100 mM NaCl, pH 7.4). AChE substrate (559 μM) was added to the sample well then incubated with the enzyme for 240 s. Immediately after incubation, a single, 20 μL aliquot was removed from the sample well and spotted onto the surface of the screen-printed electrode covering the working electrode, counter electrode and reference electrode (Fig. 1). DPV was then applied from −0.10 V to 0.80 V and the peak current intensity resulting from TCH oxidation (approx. 0.16 V) was measured for the uninhibited control, inhibitor-treated samples as well as a sample black (absent of AChE substrate). Correcting for the peak current intensity measured at 0.16 V for the blank sample, AChE activity was determined by relative comparison of peak current for the uninhibited control vs inhibitor-treated enzyme, modified from Eq. S1.

2.4. Animal tissue preparation

All experimental protocols were approved by Animal Care Committee of Sunnybrook Research Institute and followed the policies and guidelines outlined by the Canadian Council on Animal Care. At 8 weeks postnatal, (C57BL/6) mice were deeply anesthetized using ketamine (150 mg/kg) and xylazine (10 mg/kg) and perfused intracardially with 0.9% saline for 5 min at a flow rate of 10 ml/min. The brain was extracted followed by dissection of the anterior cortical regions. The collected brain tissue was flash frozen using liquid nitrogen and subsequently stored at −80°C. Homogenization of tissue samples was performed in 1:10 wt/vol cold lysing buffer (20 mM Tris·Cl, 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, pH 7.4) containing protease inhibitors (Calbiochem, La Jolla, CA, USA). This resulting homogenates were centrifuged (12,000 g, 15 min, 4°C) then AChE activity assays were performed using the collected supernatant. Protein concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, IL, USA). Stock solution of homogenized anterior cortical region was prepared to a final concentration of 9 μg/mL⁻¹. Electrochemical assays of AChE activity in mouse homogenate samples required 27 μg of protein diluted into 1.48 μl mixing volume, from which 20 μl aliquots were obtained for each sample measurement.

3. Results and discussion

The activity of purified human erythrocyte-derived AChE was first determined by Ellman's colorimetric assay [28], wherein microplate wells were prepared from a mixture of the active enzyme (0.1875 U/mL⁻¹) and the colorimetric dye, DNTB (340 μM), at physiological conditions. Enzyme-catalyzed hydrolysis was initiated by the addition of substrate, ATP (550 μM) (Fig. 1a). A sulfide-disulfide reaction between DNTB and the hydrolytic product, TCH, yields a TBN ion that absorbs strongly at 410 nm (Fig. 2, inset). Accordingly, the rate of TCH formation was related stoichiometrically to the rate of TBN production determined by spectrophotometrically measuring optical density changes at 410 nm (Fig. 2), as previously demonstrated [18, 29]. Percent enzyme activity was derived for each sample by relative comparison of the initial reaction velocities of inhibitor-treated enzyme samples (Vᵢ) to an uninhibited control (V₀). The IC₅₀ profile was determined through non-linear regression of AChE activity plotted over a range of Donepezil concentrations.
Fig. 3. Inhibition plot of human erythrocyte-derived AChE percent activity vs the log of Donepezil concentration obtained by Ellman's method. The IC₅₀ value of Donepezil was determined to be 26 ± 7 nM. Error bars denote the standard deviation determined for n = 3 measurements.

(log of 10 nM to 150 nM) (Fig. 3). Using Ellman's colorimetric assay, the IC₅₀ of Donepezil for human erythrocyte AChE measured in vitro was calculated to be 26 ± 8 nM. The observed IC₅₀ was comparable to previously reported values that estimate between 14 and 23 nM [18,29]. For all inhibitor treatment conditions, AChE samples were pre-incubated with Donepezil prior to substrate addition. Interestingly, enzyme inhibition was found to be independent of the pre-incubation time period tested for 2, 5, 10, 20, and 30 min (Fig. S1). Consequently, a fixed pre-incubation period of 10 min was applied to all subsequent sample conditions to ensure consistency between samples.

However, in view of the limitation of extrinsic colorimetric reagents, such as DTNB, we developed a label-free alternative to evaluate ChE activity through direct electrochemical oxidation of Tch on an unmodified gold electrode (Fig. 1b). A low-cost potentiostat device was implemented to modulate the potential of the gold working electrode surface (Fig. 1b and c). This compact, hand-held system was constructed to perform complex voltammetric measurements such as DPV. Measurements conducted using differentially-pulsed potentials served to minimize non-Faradaic background/current thereby improving detection sensitivity over conventional voltammetric detection systems, which commonly incorporate linearly ramped potential sweeps. In this study, we thoroughly compared the static method of direct Tch oxidation to Ellman's method, which uses kinetic sampling, as a viable alternative for characterization of AChE inhibition.

For electrochemical measurements, AChE samples were prepared as previously described for Ellman's method, except for DTNB, which was excluded from all samples. Enzyme-catalyzed hydrolysis was initiated by the addition of ATCh substrate followed by an incubation period of 4 min at 37 °C such that a sufficiently detectable concentration of Tch was produced, after which, a single-time point measurement was performed using DPV (n ≥ 3). For all DPV measurements, a 20 µL aliquot of the substrate-incubated sample was deposited onto the surface of a screen-printed electrode. The differential pulsed potential waveform was applied from 0.10 V to 0.80 V. Disposable gold screen-printed gold electrodes conveyed a number of advantages such as a significant reduction of sample of sample requirements (10–20 µL) compared to 300 µL for Ellman's method using multi-well plates). Additionally, the Tch oxidation signal was enhanced due to the high coordination affinity between the gold surfaces to the terminal thiol groups of Tch. An overview of the complete voltammetric profile for various AChE sample conditions (Fig. 4) shows the relevant peak potentials of all redox-active sample components. ATCh exhibited two anodic peaks occurring at approximately 0.39 V (II) and 0.59 V (III). Notably, Tch, was produced only in samples containing both ATCh and AChE and was defined by a single non-overlapping anodic peak at approximately 0.16 V (I) vs Ag/AgCl.

Within the applied potential window (-0.10 V to 0.80 V), Donepezil exhibited no detectable anodic peaks, however, its presence caused a distinct drop in peak current intensity at 3 suggesting the inhibited production of Tch, consistent with Fig. 1b. Accordingly, analysis over a wider range of Donepezil concentrations (10-150 nM) showed a gradual decrease in Tch peak current intensity with increasing inhibitor concentration as were previously (Fig. 5). At complete inhibition (150 nM Donepezil) the peak current at 1 saturated to a minimum current value convergent with the blank sample (dashed line), measured in the absence of ATCh substrate. Correcting all samples for the blank peak current, the characteristic Tch oxidation wave height (% Activity) was plotted against the log of Donepezil concentration (from 0 nM to 150 nM).

Fig. 4. Differential pulse voltammograms of human erythrocyte AChE samples comparing the non-overlapping redox peaks produced by ATCh (I, 0.69 V; III, 0.59 V). Samples were prepared using 0.1M Tris buffer, 300 µM ATCh and 30 nM Donepezil and measured in 50 mM PB, 100 mM NaCl, pH 7.4 on gold screen-printed electrodes vs Ag/AgCl.

Fig. 5. Differential pulse voltammograms depicting Tch oxidation at 0.35 V (I) on a gold screen-printed electrode. Samples were measured from -0.10 V to 0.80 V vs Ag/AgCl with human erythrocyte-derived AChE (1.5X10⁵ U/mg) and ATCh (550 µM) prepared in 50 mM PB, 100 mM NaCl, pH 7.4. Notable decreases in peak current intensity for each electrochemical oxidation were observed as Donpezil concentration increased. The inset shows a plot of percent AChE activity, derived from Eq. 3, vs the log of inhibitor concentration for determination of the IC₅₀ values by non-linear regression analysis. The IC₅₀ value of Donepezil was determined to be 28.17 nM (n = 3).
AChE activity was determined from Eq. S1 by substituting reaction velocity, 'v', with the corrected peak current intensity, 'i'. Using nonlinear regression analysis of AChE activity plots (Fig. 5, inset), the IC50 of Donepezil determined by DPV was calculated to be 28 ± 7 nM relative to the value of 24 ± 8 nM obtained for Elman's method. The comparable IC50 and reproducibility of the data suggested that the presented method for electrochemical TCh oxidation posed a suitable alternative for preliminary profiling of ChE activity.

Due to the notably low oxidation potential of TCh (0.15 V), no interfering peak potentials were observed in vitro. In particular, AChE contains tyrosine, tryptophan and cysteine, which are inherently electroactive but generated only a single anodic peak at approximately 0.40 V (Fig. 5) [31,32]. Although the ATCh substrate contains a sulfur group, it exhibits two overlapping peaks at much higher potentials of 0.40 V and 0.60 V compared to TCh. Thus, in order to establish selectivity of the unmodified gold electrode system for TCh detection, electrochemical profiles were measured for a number of potentially interfering sulfur-containing biological compounds: glutathione, homocysteine, cysteine and methionine (Fig. S5). Like TCh, glutathione, cysteine and homocysteine pose a free thiol subunit (SH), whereas the sulfur group of methionine is constituted between two alkyl chains (R-S-R). Cysteine and homocysteine exhibited anodic peaks at approximately 0.55 V and 0.48 V respectively. The oxidation of glutathione was not observed over the potential range of -0.10 to 0.80 V, whereas methionine yielded a single anodic peak occurring at 0.80 V. All sulfur-containing compounds were found to undergo oxidation at higher potentials compared to TCh allowing for sufficient peak potential discrimination [33].

To further address concerns of potentially interfering redox active materials and validate the applicability of this system in biological samples, additional activity assays were performed on AChE-containing homogenates from the anterior cortex of C57BL/6 mice. Control measurements of diluted homogenates showed a single, broad anodic peak at approximately 0.80 V (vs Ag/AgCl) attributed to the wide mixture proteins containing redox-active amino acids (tyrosine, tryptophan or cysteine) present within crude samples (Fig. S6) [34]. No overlapping peaks were observed at the TCh oxidation potential of 0.16 V, though in the presence of ATCh, a cathodic shift in the previously observed peak potentials was detected both for ATCh (0.35 V and 0.55 V) as well as TCh (0.07 V) (Fig. 6) due to the presence of surfactants required in the preparation of homogenate samples. Ascorbic acid (AA) undergoes a well-characterized oxidation process on gold electrodes close to 0.15 V vs Ag/AgCl [35,36] leading to a potential source of interference for TCh oxidation (Fig. S3). Notably, no AA oxidation was observed in homogenate controls (Fig. 6, dashed line). We assert that the absence of the AA peak is due to its low concentration relative to AChE activity. The concentration of AA in the anterior cortex of mice was approximately 0.38 ng·μL⁻¹ protein (2.17 μM·1 protein) [37], with similar concentrations of AA observed in other rodent [38,39]. The total quantity of C57BL/6 anterior cortex protein required to conduct each AChE assay was 27 μg (10 ng AA) prepared in a total mixing volume of 48 μL (1 μM AA) from which 20 μL aliquots were removed for electrochemical characterization. This low concentration of AA relative to high activity of AChE allowed for the TCh oxidation peak to be detected within the same potential window of approximately 0.15 V without interference. This observation was further substantiated from the analysis of pure AA (1 μM) (Fig. S3, inset), which demonstrated no observable oxidative process.

Donepezil-treated homogenates still exhibited a characteristic drop in peak current intensity at 0.07 V with increasing inhibitor concentration, which was confirmed subsequently by Elman's method. Although both detection methods were noted to demonstrate a higher standard deviation (Fig. 6, inset) when compared to purified human erythrocyte AChE, inhibition of enzyme activity at 50% was found to be significant using DPV in mouse homogenate samples (P<0.01 by one-way ANOVA, n=3).

4. Conclusions

In this study, the merits of a low-cost, label-free electrochemical detection platform were explored in contrast to the conventional Elman's colorimetric assay for the characterization of AChE activity. The high similarities in enzyme inhibition profiles determined from comparative studies promote the utility of this electrochemical detection system as an alternative technique for simple and efficient screening of novel ChE inhibitors as therapeutic agents. Current limitations to high-throughput analysis are expected to be overcome through further development of a multi-channel potentiostat system in future work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.aca.2013.02.031.

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