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The role of choline acetyltransferase variants in Alzheimer's disease models

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Graduate Program in Physiology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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THE ROLE OF CHOLINE ACETYLTRANSFERASE VARIANTS IN ALZHEIMER’S DISEASE MODELS

(Thesis format: Monograph)

by

Shawn Albers

Graduate Program in Physiology

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

The primate specific 82-kDa choline acetyltransferase (ChAT) isoform is found in the nuclei of cholinergic neurons, with a disruption in the subcellular localization in aging and AD brain samples. The functional significance of this protein is poorly understood. Previous studies have revealed a potentially protective role for 82-kDa ChAT, mediated through a reduction in amyloid-β_{1-42} (Aβ_{1-42}) release in APP/PS1 double transgenic primary cortical neurons. Here we examine the effect of 82-kDa ChAT expression in transgenic neurons, on the amyloidogenic processing of amyloid precursor protein (APP) and Aβ production. In this study we demonstrate 82-kDa ChAT transcriptionally increases golgi-localized γ-ear-containing ARF-binding 3 (GGA3), a protein that traffics BACE1 to the lysosome for degradation. Increased GGA3 expression coincides with a reduction in BACE1 protein and activity levels, which may account for the reduction in Aβ_{1-42} production. These findings indicate a potential regulatory function of 82-kDa ChAT, specifically at the genetic level.

Keywords

Choline acetyltransferase, Alzheimer’s disease, golgi-localized γ-ear-containing ARF-binding, β-site APP cleaving enzyme
Co-Authorship

Studies described in this thesis were performed by Shawn Albers, with the following assistance:

1) Daisy Wong assisted in conducting ChAT activity assays
2) Daisy Wong, Ewa Jaworski, and Meera Karajgikar assisted in breeding of the mice colonies and preparation of primary neuron cultures
3) Daisy Wong assisted in confocal microscopy
4) Fatima Abji carried out the Aβ ELISAs in Figure 3.2
5) Dr. R.J. Rylett designed Figure 1.2

All experiments, excluding Figure 3.2, were carried out by Shawn Albers in the laboratory of Dr. R.J. Rylett. The thesis was written by Shawn Albers.
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<tr>
<td>Acetyl-CoA</td>
<td>Acetyl coenzyme A</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ADAM10</td>
<td>A disintegrin and metalloproteinase 10</td>
</tr>
<tr>
<td>APOE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid-β</td>
</tr>
<tr>
<td>BACE1</td>
<td>β-site APP cleaving enzyme</td>
</tr>
<tr>
<td>C83</td>
<td>C-terminal fragment 83</td>
</tr>
<tr>
<td>C99</td>
<td>C-terminal fragment 99</td>
</tr>
<tr>
<td>ChAT</td>
<td>Choline acetyltransferase</td>
</tr>
<tr>
<td>CHT</td>
<td>Choline transporter</td>
</tr>
<tr>
<td>cNLS</td>
<td>Classical nuclear localization signal</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DIV</td>
<td>Days \textit{in vitro}</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced ChemiLuminescence</td>
</tr>
<tr>
<td>EGRF</td>
<td>Epithelial growth factor receptor</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>G-418</td>
<td>Geneticin</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GGA</td>
<td>Golgi-localized ( \gamma )-ear-containing ARF-binding</td>
</tr>
<tr>
<td>H(_2)O(_2)</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney cells</td>
</tr>
<tr>
<td>HMW</td>
<td>Higher molecular weight</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase-conjugated</td>
</tr>
<tr>
<td>Ifu</td>
<td>Infectious units</td>
</tr>
<tr>
<td>MCI</td>
<td>Mild cognitive impairment</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear export signal</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>NeuN</td>
<td>Neuronal nuclear protein</td>
</tr>
<tr>
<td>NFTs</td>
<td>Neurofibrillary tangles</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-asparate</td>
</tr>
<tr>
<td>NTG</td>
<td>Non-transgenic</td>
</tr>
<tr>
<td>OD&lt;sub&gt;260&lt;/sub&gt;</td>
<td>Optical density 260 nm</td>
</tr>
<tr>
<td>PS1</td>
<td>Presenilin 1</td>
</tr>
<tr>
<td>PS2</td>
<td>Presenilin 2</td>
</tr>
<tr>
<td>PrP</td>
<td>Prion protein</td>
</tr>
<tr>
<td>PUFAs</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Real time polymerase chain reaction</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative fluorescence units</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SORT1</td>
<td>Sortilin 1</td>
</tr>
<tr>
<td>TG</td>
<td>Transgenic</td>
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<table>
<thead>
<tr>
<th>Acronym</th>
<th>Name</th>
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<tr>
<td>TGN</td>
<td>Trans-Golgi network</td>
</tr>
<tr>
<td>VACHT</td>
<td>Vesicular acetylcholine transporter</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 Alzheimer’s disease

Alzheimer’s disease (AD) is a progressive neurological disorder and the leading cause of age-related cognitive disability, characterized by a loss of memory and cognitive function. AD currently accounts for 50%-80% of the total cases of dementia among patients over the age of 65. The prevalence of AD is continuing to rise with the increase of the aging population, creating financial pressure on the healthcare system. It is predicted that by 2050 the annual cost of caring for individuals with AD will increase from $203 billion to over $1.2 trillion in the USA alone. In a report published by the Alzheimer Society of Canada entitled “The Rising Tide”, the magnitude of the problem faced by the Canadian health care system and society of caring for individuals with age-related diseases was presented. It is predicted that by the year 2030, the number of Canadians with AD will be at least 1.1 million, costing at least $153 billion dollars per year to provide care to these individuals, and involve at least 756 million hours of informal caregiving time for families and other unpaid helpers.

Patients with the disease live an average of 10 to 12 years after symptoms become noticeable, but the duration of the disease can range from 4 to 20 years, depending on the diagnosis and management. An increasing number of individuals are being diagnosed with early-onset or familial AD, where symptoms
are seen by patients 40 to 50 years of age. Familial forms of AD are linked to mutations in either amyloid precursor protein (APP), or presenilin 1 (PS1) and presenilin 2 (PS2), components of the γ-secretase complex (Octave et al., 2008), resulting in elevated β-amyloid peptide (Aβ) levels or altered Aβ production favouring the release of toxic Aβ1-42 (Citron et al., 1992, Jankowsky et al., 2004). Inheritance of the ε4 allele of apolipoprotein E (APOE) increases the risk for developing dementia and lowers the age of onset of the sporadic form of AD. The mechanism by which this mutation affects the risk and age of onset of AD is currently unknown, but the prevailing evidence suggests that it is related to increased Aβ aggregation and decreased Aβ clearance (Kim et al., 2009).

1.2 Etiology of AD

The initiating cause of AD is still unknown, but the pathogenesis of the disease has long been linked to the formation of the ~4-kDa Aβ, its deposition as amyloid plaques, and to intracellular neurofibrillary tangles (NFTs). Research into the APP processing pathway and the link between mutations in the PS1 genes has lead to the amyloid hypothesis becoming a main focus of AD research. More recent studies have only strengthened the association between the amyloid hypothesis and the prevalence of AD. First, large depositions of hyperphosphorylated tau protein in NFTs are also found in brains of patients diagnosed with Parkinson’s disease, with this being caused by mutations in the gene encoding the tau protein and resulting in profound neurodegeneration.
These patients do not have deposition of amyloid plaques and display few of the other characteristics of AD, demonstrating a fundamental role for Aβ in AD pathogenesis (Hardy and Selkoe, 2002). Mutations in tau are also shown to cause autosomal dominant frontotemporal dementia as well as amyotrophic lateral sclerosis (Poorkaj et al., 2001). Second, transgenic mice that express both mutant human tau and mutant human APP show increased levels of tau-positive tangles compared to mice that express only mutant human tau. These findings suggest that either APP or Aβ influences the formation of tau-positive NFTs and are present prior to tangle deposition (Lewis et al., 2001). Finally, genetic variability in Aβ breakdown and removal can contribute to the risk of late-onset AD (Ertekin-Taner et al., 2000)

Amyloid plaques are formed by the aggregation of 37-43 amino acid fragments of Aβ within the brain parenchyma (De Strooper and Annaert, 2000). Aβ is produced by an amyloidogenic processing pathway that involves sequential enzymatic cleavage of the APP carried out by the membrane-bound β-site APP-cleaving enzyme β-secretase (BACE1) and by the γ-secretase complex enzymes. APP is first cleaved by BACE1 to produce a secreted sAPPβ polypeptide and a membrane bound C-terminal fragment (C99) (Vassar et al., 1999). The γ-secretase complex then further cleaves C99 resulting in the secretion of Aβ. Conversely, Aβ production can be interrupted by the non-amyloidogenic pathway, in which APP is cleaved within the Aβ sequence by α-secretase thereby releasing soluble APPα and a membrane bound C-terminal fragment
Figure 1.1 APP processing through the amyloidogenic and non-amyloidogenic pathway. Cleavage of APP in the amyloidogenic pathway involves processing by both β-secretase (BACE1) and the γ-secretase complex which releases either a 40 or 42 residue Aβ peptide. The Aβ peptides begin to aggregate forming soluble oligomers and overtime result in the formation of amyloid plaques associated with Alzheimer’s disease. Alternatively, APP processing can occur through the non-amyloidogenic pathway, by which it is first cleaved by α-secretase (ADAM10) and the γ-secretase complex, avoiding the formation of toxic Aβ peptides.
(C83) (Vassar et al., 1999). The γ-secretase complex further cleaves C83 resulting in the secretion of nonpathogenic p3 fragment (Figure 1.1).

The direct mechanisms by which extracellular Aβ exerts toxic effects on cholinergic neurons are unknown, but Aβ aggregates are believed to severely alter cholinergic synaptic transmission by reducing ACh synthesis and release (Kar et al., 2004). Nunes-Tavares et al (2012) investigated the effect of Aβ oligomers on cholinergic neurons and found a major reduction in ChAT activity, with no change in neuronal viability or total ChAT protein expression. Aβ-induced oxidative stress appears to play a predominant role in this inhibition, which appears to be mediated through an imbalance between reactive oxygen species (ROS) production and antioxidant defense mechanisms. Aβ oligomers are able to increase ROS levels in cholinergic neurons through a mechanism requiring N-methyl-D-aspartate (NMDA) receptor activation (De Felice et al., 2007). Consistent with this finding, both treatment with antioxidant polyunsaturated fatty acids (PUFAs) and the NMDA receptor inhibitors MK-801 and memantine prevents ChAT inhibition (Nunes-Tavares et al., 2012). Further study has shown that insulin signaling down-regulates Aβ oligomer binding of the NMDA receptor which resulted in a reduction of both ROS production and ChAT inhibition (Nunes-Tavares et al., 2012). These results demonstrate a significant role of Aβ-induced ROS toxicity on ChAT inhibition and cholinergic function in AD patients.
1.3 **GGA3 and BACE1 trafficking**

The Golgi-localized γ-ear-containing ARF-binding (GGA) family of proteins are ubiquitously expressed and are involved in the trafficking of proteins from the trans-Golgi network to the endosome/lysosome system (Ghosh and Kornfeld, 2004). Currently, there have been three GGAs discovered in mammalian cells (GGA1, GGA2, and GGA3) (Boman et al., 2000). GGAs contain four distinct domains: an N-terminal VHS domain (VPS27, Hrs, and STAM) that binds the acidic di-leucine DXXLL motif; a GAT domain (GGA and Tom1) that binds Arf:GTP; a hinge region that binds clathrin; and a COOH-terminal γ-adaptin ear region which functions to recruit accessory proteins (Kang et al., 2010). GGAs mediate trafficking through the recognition of specific signal motifs. For example, proteins that have been modified with the addition of mannose 6-phosphate groups bind to the mannose 6-phosphate receptor (MPR) and are bound at the VHS domain by GGAs through the recognition of the DXXLL motif located on the carboxyl-terminal of the MPR (Johnson and Kornfeld, 1992). A key component of the GGA trafficking pathway mediated through the DXXLL-VHS interaction are the acid hydrolases that are shuttled to the lysosome (Doray et al., 2002).

BACE1 is a 501 amino acid type 1 transmembrane protein that is ubiquitously expressed, with the highest level of mRNA and protein levels found in neurons in the brain (Citron, 2004). As a member of the aspartyl protease family, it contains an active protease site on the luminal side that has the ability to cleave APP at the β-secretase cleavage site. This process is enhanced within intracellular compartments of the secretory pathway, specifically the Golgi and
endosomes, where the acidic pH optimizes BACE1 activity (Vassar et al., 1999). Transport of BACE1 is mediated through the C-terminal fragment di-leucine (DXXLL) sorting signal that is present on many transmembrane proteins.

The GGA3 GAT domain has been reported to bind ubiquitin and directly facilitate the delivery of activated epithelial growth factor receptor (EGFR) to lysosomes (Puertollano and Bonifacino, 2004). RNAi silencing of GGA3, but not GGA1 or 2, results in the accumulation of EGRF that demonstrates a GGA3-specific trafficking mechanism. This GGA3-mediated lysosomal trafficking has also been reported to regulate BACE1 degradation. BACE1 has been shown to be ubiquitinated at Lys-501, undergoing both monoubiquitination and also Lys-63-linked polyubiquitination that facilitates its transport to and degradation in the lysosomal system (Kang et al., 2010). GGA3 is able to recognize and bind directly with ubiquitinated BACE1 and target it for lysosomal degradation. This process is independent of the GGA3 VHS domain or the BACE1 di-leucine motif, as mutations in both these domains did not abrogate their binding. Further support of this interaction was demonstrated when Kang et al (2010) reported BACE1 accumulation in early endosomes in H4 cells depleted of GGA3, compared to control cells.

Several studies have focused on the expression and activity of BACE1 mRNA and protein levels in AD brains. It has been shown that BACE1 mRNA levels remained unchanged (Tan and Evin, 2012), but there are significant increases in BACE1 protein and activity levels, particularly in the temporal cortex (Fukumoto et al., 2002; Kang et al., 2010). The increase in BACE1 protein may
be associated with various post-translational modifications including the removal of the pro-peptide, glycosylation, and phosphorylation (Li et al., 2006). Conversely, in these brain regions with high levels of BACE1 expression, GGA3 levels were significantly reduced (Tesco et al., 2007). There is growing evidence that BACE1 is a stress-induced protein, whereby elevated levels can be seen in rodents following cerebral ischemia and traumatic brain injury (Walker et al., 2012). Caspase-3 activation following these insults has been shown to elevate BACE1 protein levels and activity though post-translational stabilization of BACE1 (Tesco et al., 2007). Moreover, GGA3 is also a substrate of caspase-3 resulting in its cleavage and inactivation, thereby causing the increased BACE1 levels and activity and enhanced amyloidogenic processing of APP (Tesco et al., 2007).

1.4 Cholinergic Neurons

The cholinergic hypothesis of AD implicates degeneration of both the neurons and synapses that produce and release acetylcholine (ACh) as a major cause of the cognitive impairment observed in AD patients. There appear to be changes in ACh production in brain of AD patients related to impaired transport of choline into cholinergic nerve terminals by the choline transporter (CHT) and synthesis by the enzyme choline acetyltransferase (ChAT). A decline in ChAT activity and loss of ChAT gene expression is found in regions affected by the disease (Bird et al., 1983, Zambrzycka et al., 2002).
Cholinergic neurons are an integral part of the mammalian brain, and play an important role in cognitive function, including memory formation through the action of the neurotransmitter ACh (Coyle et al., 1983, Francis et al., 1999). Modest changes in cholinergic neuron function occur during normal aging, and these changes are accentuated in neurological diseases such as AD and related dementias. Although the degeneration of cholinergic neurons does not cause AD, it plays a primary role in the clinical symptoms of AD. Cholinergic neuron projections from the nucleus basalis of Meynert to the cortex and hippocampus are particularly damaged in AD patients (Schliebs and Arendt, 2011). Dysregulation of the cholinergic signaling cascade involving the expression and function of the major cholinergic proteins, decreased ChAT activity is an early hallmark of AD (Nunes-Tavares et al., 2012). While there is no cure for AD, a number of therapeutic interventions, both current and in the past, are focused on treating the cholinergic neurotransmitter deficits. Early treatments focused on the replacement of ACh precursors (choline or lecithin), but these failed to increase overall cholinergic activity (Francis et al., 1999). A more recent and well developed therapeutic approach focuses on the use Aricept (Pfizer), Exelon (Novartis) and Reminyl (Janssen), which are inhibitors of the enzyme acetylcholinesterase (AChE). These drugs work by decreasing the activity of AChE, thereby preventing the hydrolysis of ACh to choline and acetate and prolonging the existence and effect of ACh at the synapse.
1.5 Choline Acetyltransferase

ACh, a critical mediator of neuronal communication, is produced in cholinergic neuronal cytoplasm including nerve terminals by the ChAT catalyzed combination of acetyl coenzyme A (acetyl-CoA) and choline. ACh is transported into synaptic vesicles by the vesicular acetylcholine transporter (VACHT) and, upon neuronal depolarization, these vesicles fuse with the plasma membrane to release ACh into the synaptic cleft where it binds to post-synaptic receptors. AChE hydrolyzes ACh in the synaptic cleft to choline and acetate, from where choline is immediately recycled back into the presynaptic terminal by the sodium-coupled high-affinity CHT to serve as substrate for ACh re-synthesis.

Currently, seven isoforms of ChAT mRNA have been characterized in humans: R1/2-, N1/2-, H-, S-, and M-transcripts (Gill et al., 2007) (Figure 1.2). All human ChAT mRNAs translate a 69-kDa protein, but the S- and M-transcripts also encode a 74- and 82-kDa isoform, respectively (Oda et al., 1992). Based on immunohistochemical localization studies involving human necropsy tissues, it has been reported that both 82-kDa and 69-kDa ChAT are expressed in cholinergic neurons in the spinal cord, basal forebrain and putamen, as well as an 82-kDa ChAT specific expression in the clastrum (Gill et al., 2007). The 69-kDa enzyme is predominately localized to the cytoplasm of cholinergic neurons, whereas the 82-kDa ChAT is found predominately in the nucleus. The 82-kDa ChAT protein has two nuclear localization signals (NLSs), one of which is located within its 118-amino acid residue amino-extension and one of which is in common with the 69-kDa sequence, and this facilitates
The cholinergic gene locus encodes multiple ChAT proteins:

- **69-kDa** ChAT protein
- **82-kDa** ChAT protein

Figure 1.2. The cholinergic gene locus. The cholinergic gene locus contains the coding sequences for multiple ChAT exons and VACHT. The human specific M-ChAT transcript variant has two functional translation initiation sites resulting in production of both 69- and 82-kDa ChAT proteins. The 82-kDa ChAT protein has an extended amino terminus which contains a nuclear localization signal.
trafficking of the protein through the nuclear pore complex (Gill et al., 2003). Classical NLS (cNLS) sequences are rich in both of the basic amino acids arginine and lysine (Tachibana et al., 1999). Proteins in the cytoplasm containing a cNLS sequence can be bound by the heterodimeric import receptor, importin α/β, which facilitates their translocation through the nuclear pore (Lange et al., 2007). The NLS sequence found in the amino-terminal extension of 82-kDa ChAT contains a basic region that is both necessary and sufficient for nuclear localization of the enzyme (Resendes et al., 1999). Nuclear export of 69-kDa ChAT and a small pool of 82-kDa ChAT is mediated through the recognition of nuclear export signals (NES). These sites are typically rich in valine, leucine and isoleucine residues (Tang et al., 1999). Both isoforms of ChAT contain NESs, but the majority of the 69-kDa ChAT nuclear export is mediated by the leptomycin B-sensitive Crm-1 export pathway (Gill et al., 2003).

The subcellular localization of 82-kDa ChAT appears to change with increasing age and in individuals diagnosed with mild cognitive impairment (MCI). It was reported that there is an age-related alteration of the subcellular distribution of 82-kDa ChAT, whereby individuals over the age of 80 years had increased levels of cytoplasmic 82-kDa ChAT and decreased levels of nuclear localized 82-kDa ChAT. Furthermore, this change in the subcellular localization of 82-kDa ChAT expression is accentuated in MCI and AD patients. Currently, the mechanism underlying the altered subcellular localization of 82-kDa ChAT in cholinergic neurons during aging and in individuals diagnosed with MCI or early AD is unknown. However, it is clear that any situation that alters ChAT activity
will directly impact ACh synthesis and may result in reduced cholinergic neurotransmission.

Previous work in our laboratory has focused on the role of nuclear 82-kDa ChAT and how it relates to changes in gene expression and regulation. Through microarray analysis, it was observed that ChAT expression resulted in a change in the expression on several APP processing-related genes. Taken together, the relationship between the changes in ChAT-mediated expression of these genes led to a prediction of reductions in both BACE1 activity and Aβ production. More recently, a study has focused on a secondary function of nuclear localized ChAT, as a possible regulator of gene transcription. Matsuo and coworkers (2010) reported that overexpression of 82-kDa ChAT enhanced transcriptional activation of the *CHT1* gene in SH-SY5Y human neuronal cells. The mechanism behind the transcriptional regulation by 82-kDa ChAT is still unknown, but there are multiple possibilities by which this regulation could occur. Nuclear ChAT maintains its enzymatic activity and could result in the production of ACh in the nucleus and activate transcription through muscarinic ACh receptors. Interestingly, both G-protein-coupled muscarinic ACh receptors (Lind and Cavanagh, 1993) and nicotinic ACh receptors (Okuda et al., 1993) have been detected in the nuclear envelope, however, these receptors are predicted to have ligand binding sites that face the luminal side of the nuclear envelope, so nuclear-synthesized ACh would not likely be able to successfully bind to these receptors (Matsuo et al, 2011). A second possibility is that 82-kDa ChAT may bind directly to certain DNA regions and act as a transcription factor. Based on
analysis of the three-dimensional structure of ChAT (Kim et al., 2006), the protein displays basic patches along its surface that are predicted to be capable of binding DNA. The ability of 82-kDa ChAT to bind to DNA either directly or in association with other proteins or nuclear components has not yet been examined.

1.6 Transgenic Mouse Models of AD

The use of genetically-modified mice as a model of neurodegenerative diseases can give a unique insight into the progression of these diseases. Early transgenic models of AD were based on overexpression of APP to promote increased levels of Aβ deposits (Andra et al., 1996), but the progression of these deposits were slow, needing at least 10 months to develop amyloid plaques or deficits in memory and learning. More recent studies have involved the development of transgenic mice that express a gain-of-function mutant form of PS1 (Duff et al., 1996), but these mice also displayed very limited Aβ pathology or cognitive impairment. One of the most promising commercially-available murine models of AD is the double transgenic APPswe/PS1dE9 mouse. These mice were generated with mutant transgenes for APP (APPswe: KM594/5NL) and PS1 (dE9: deletion of exon 9), driven by mouse prion protein (PrP) promoter elements, which direct transgene expression within central nervous system (CNS) neurons. Plasmids containing each transgene were coinjected into B6C3HF2 pronuclei and insertion occurred at a single locus. The Swedish APP
mutant allows for greater $\mathrm{A}\beta$ production by promoting processing by the beta-secretase pathway, while the PS1 mutant promotes $\mathrm{A}\beta_{1-42}$ production. These mice have significant $\mathrm{A}\beta$ deposition beginning around 4 months with plaque formation by 6 months (Garcia-Alloza et al., 2006).

1.7 Rationale

1) Previous work has demonstrated that overexpression of either 82-kDa human ChAT or mutant R560H 82-kDa ChAT in cortical neurons derived from APP/PS1 transgenic mice reduces the levels of $\mathrm{A}\beta_{1-42}$ released into the culture media when compared to control neurons expressing GFP. Further work has demonstrated that neuronal expression of 82-kDa ChAT can alter expression of APP processing genes and levels of APP processing proteins, leading to reduced overall BACE1 protein levels and ultimately lowering $\mathrm{A}\beta_{1-42}$ production. These previous studies demonstrate a novel role for 82-kDa ChAT, but the exact mechanism of 82-kDa ChAT function was not investigated. The goal of this study will identify the cellular pathway through which 82-kDa ChAT reduces the level of $\mathrm{A}\beta_{1-42}$ release from APP/PS1 mouse cortical neurons.

2) $\mathrm{A}\beta_{1-42}$ toxicity is in part mediated by elevating cellular ROS levels which leads to cellular dysfunction and eventual cell death. AD patients have a marked increase in ROS levels and this is believed to contribute to the neurodegeneration observed in the later stages of the disease. Cysteine
(Cys) residues are particularly susceptible to oxidative toxicity, resulting in the formation of disulfide bonds both within and between proteins. The 82-kDa ChAT protein has a higher than typical number of Cys residues, and this could make the protein vulnerable and potentially readily oxidized by ROS. The goal of this study is to investigate the effect of varying ROS agents and concentrations on both the structure and subcellular localization of 82-kDa ChAT.

1.8 Study: Effect of 82-kDa ChAT on Aβ production

Hypothesis:

1) Expression of nuclear 82-kDa ChAT in APP/PS1 transgenic neurons regulates the expression of APP processing proteins, thereby promoting the non-amyloidogenic pathway.

2) Exposure of neural cells to Aβ or oxidative stress causes alterations in both 69- and 82-kDa ChAT structure, function, and subcellular localization.

Specific Aims:

1) Determine the cellular mechanism that underlie the reduction in Aβ levels in cortical neurons cultured from APP/PS1 transgenic mice that ectopically express 82-kDa ChAT.

2) Identify the effects of Aβ and oxidative stress on both 69- and 82-kDa ChAT structure, function, and localization.
CHAPTER TWO: MATERIALS AND METHODS

2.1 Materials

The majority of the general laboratory chemicals used in these studies were purchased from Sigma-Aldrich (St. Louis, MO, USA) and BioShop (Burlington, ON, Canada). HEK293 and SH-SY5Y cells were acquired from American Type Culture Collection (Manassus, VA, USA) and SN56 cells were a gift from Dr Bruce Wainer (Emory University, Oxford, GA, USA). Cell culture reagents: Dulbecco’s modified eagle medium (DMEM), minimal essential medium (MEM), and fetal bovine serum (FBS) were supplied by Invitrogen (Burlington, ON, Canada). The REDExtract-N-Amp Tissue PCR Kit for genotyping and GeneElute Kit for RNA isolation were purchased from Sigma-Aldrich. BioRad (Mississauga, ON, Canada) supplied the iScript cDNA synthesis kit and iQ SYBR Green kit. The β-secretase Activity Kit was from BioVision (CA, USA) and the LightSwitch Luciferase assay system was purchased from SwitchGear Genomics (Menlo Park, CA, USA). The human Aβ_{1-42} ELISA kit was purchased from Invitrogen (Burlington, ON, Canada). Primary antibodies used for immunoblots include anti-actin (1-19) and anti-ubiquitin (P4D1) were purchased from SantaCruz Biotechnology Inc. (Santa Cruz, CA, USA), anti-GGA3 and anti-myc from Cell Signaling (MA, USA), anti-Flag from Sigma-Aldrich, anti-APP from Millipore (Billerica, MA, USA), anti-BACE1 (C-term) from Abcam (Cambridge, MA, USA) and secondary anti-rabbit and anti-mouse horseradish peroxidase-conjugated
(HRP) antibodies from Jackson Laboratories (MA, USA). For immunoprecipitation, anti-BACE (C-term, clone 61-3E7) was purchased from Millipore and for immunocytochemistry, anti-NeuN (A60) and secondary AlexaFluor conjugated antibodies were purchased from Millipore and Invitrogen respectively. Enhanced ChemiLuminescence (ECL) immunoblot reagents were obtained from GE Healthcare Life Sciences (Baie d’Urfé, QC, Canada) and Clarity Western ECL substrate from BioRad. GFP and GGA3 shRNA lentiviral particles were purchased from SantaCruz Biotechnology Inc.

2.2 Culture of Cell Lines

Multiple cell lines were used throughout this project, including HEK 293 (human embryonic kidney cells), Neuro-2A (N2A, mouse brain neuroblastoma cells) SH-SY5Y (human neuroblastomas cells) and SN56 (rat medial septal neuron immortalized by fusion with a mouse myeloma cell line) cells, which were grown at 37°C and 5% CO₂ in a humidified environment. HEK 293 and N2A cells were maintained in MEM supplemented with 10% FBS and 0.1% gentamycin. SH-SY5Y and SN56 cells were grown in DMEM supplemented with 10% FBS and 100 µg/mL penicillin-streptomycin. SH-SY5Y cells transfected with a plasmid encoding 82-kDa ChAT using Lipofectamine 2000 were selected for stable transformants with 500 µg/mL G-418 (Geneticin) for 4 weeks. Stably-expressing 82-kDa ChAT SH-SY5Y cells were maintained in 100 µg/mL G-418.
2.3 Transgenic Mice and Genotyping

Heterozygous double transgenic male mice expressing \( \text{APP}_{\text{swe}} \) and \( \text{PS1dE9} \) in a C57/Bl6 background were bred with non-transgenic (NTG) female C57/Bl6 mice (Jackson Laboratories). The mouse colonies were maintained on a 12-hour light and dark schedule, and all procedures were approved by the Animal Use Subcommittee at Western University in accordance with the guidelines set out by the Canadian Council on Animal Care.

Pregnant females were sacrificed at gestational day 15-16 and genotyping of embryos was carried out using REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich). Tail clippings from embryos were digested and DNA was extracted for PCR amplification using primers (Table 2.1) specific for the two transgenes and endogenous mouse ChAT gene promoter (FLOXG) as an internal control. The amplified DNA was loaded onto a 1% agarose gel, and following electrophoresis amplicons were visualized using Redsafe nucleic acid stain (iNtRON).

Table 2.1: Primers used for genotyping of APP/PS1 transgenic embryos

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Amplicon Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLOXG</td>
<td>Forward: GAGAGTACTTTGCTGGGAGGA&lt;br/&gt;Reverse: GGCCAGAGTAAGACCTCCCTTG</td>
<td>171 bp</td>
</tr>
<tr>
<td>( \text{APP}_{\text{swe}} )</td>
<td>Forward: GACTGACCACCTCGACCAGGTTCTG&lt;br/&gt;Reverse: CTTGTAAGTTGGATTCTCATATCCG</td>
<td>350 bp</td>
</tr>
<tr>
<td>PS1dE9</td>
<td>Forward: AATAGAGAACGGCAGGAGCA&lt;br/&gt;Reverse: GCCATGAGGGCACTAATCAT</td>
<td>608 bp</td>
</tr>
</tbody>
</table>
2.4 Cortical Neuron Cultures

Brains were removed from embryonic day 15 mouse embryos and cortices dissected. Tissues were dissociated with trypsin at 37°C and triturated in a DNAse solution with chicken egg white trypsin inhibitor. Cells were suspended in Neurobasal medium supplemented with B_{27}, N_{2}, penicillin/streptomycin and L-glutamine, then plated on poly-L-ornithine coated 35-mm (1.6 x 10^6 cells/plate) and 12-well (5 x 10^5) plates and maintained in culture for 8 days in vitro (DIV).

In some experiments, cortical neurons seeded on 35 mm culture plates were treated with 25 µM chloroquine (Sigma) or vehicle for 18 hours prior to experimentation.

2.5 ChAT-encoding Adenovirus Synthesis and Purification

Full-length human 82-kDa ChAT cDNA containing an EcoRI (GAATTC) site at the amino-terminus and BglII (GATCT) site at the carboxyl-terminus was ligated into the pDC316 shuttle vector which contained the loxP site necessary for recombination in the adenoviral genome. HEK293 cells were co-transfected with the shuttle vector and the adenoviral genomic plasmid (Ad5 E1-deleted adenovirus). Using Cre recombinase catalyzed site-specific recombination at the loxP sites described by Ng et al. (1999) active adenovirus stocks were generated. Virus expressing GFP and catalytically-inactive R560H 82-kDa ChAT were also prepared.
Adenoviral stocks were amplified for 10 DIV in HEK293 cells that provide the E1 gene necessary for viral replication. Cells were harvested and all adenovirus was collected following 3 freeze-thaw cycles in 0.1M Tris-HCL (pH 8.0). Active viral particles were purified through cesium chloride gradients, aliquoted and stored at -80°C. Viral particle concentrations were determined with a NanoDrop spectrophotometer at the optical density 260 nm (OD\textsubscript{260}) using the formula infectious particles/mL = OD\textsubscript{260} x 10^{12}. The multiplicity of infection (MOI), the number of infectious viral particles per cells plated, was calculated and it was determined that an MOI of 100 produced the highest number of adenovirus expressing cortical neurons without apparent toxicity.

2.6 Lentiviral Transduction of Cultured Neurons

Mouse cortical neurons were maintained at 37° and 5% CO\textsubscript{2} in a humidified environment for 3 DIV prior to infection with lentivirus encoding shRNA targeting GGA3 transcripts (Santa Cruz). Conditions for infection of neuron cultures were optimized on a 12-well plate for cell density, infection time and viral dose. Cortical neurons were seeded at 500,000 cells and infected with 10 µL of lentivirus preparations encoding either GFP control (copGFP) or shRNA specific for GGA3 knockdown with no known cross-reactivity based on sequence analysis. Subsequently, cultured neurons were transduced with adenovirus expressing either GFP, 82-kDa ChAT or R560H 82-kDa ChAT on 6 DIV for 48 hours followed by RNA and protein isolation.
2.7 Immunoblots

Cells were washed with HBSS and lysed for 30 minutes at 4°C in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 0.5% Triton X-100, 1 mM AEBSF, 10 µg/mL leupeptin, 25 µg/mL aprotinin, 10 µg/mL pepstatin A and 700 units/mL DNase 1. Protein concentrations were determined using a Bradford assay (BioRad) and bovine serum albumin as a standard. Aliquots containing 50 µg of total cell lysate protein were heated at 100°C for 5 min following the addition of one-half volume of 3X Laemmlia sample buffer (6% SDS, 0.1875 M Tris-HCl pH 6.8, 30% glycerol, 0.015% bromophenol blue, and 7.5% β-mercaptoethanol). Proteins were separated on 10% SDS-PAGE gels, then transferred to PVDF membranes. Membranes were blocked in 10% non-fat dry milk in wash buffer (TBS, 0.01% Tween-20) for 1 hour at room temperature. Membranes were incubated in primary antibody (Table 2.2) in wash buffer with 5% non-fat dry milk or 5% BSA, overnight at 4°C. Membranes were washed and incubated with horseradish peroxidase conjugated secondary antibody in wash buffer with 5% non-fat dry milk for 1 hour at room temperature. Immunoreactive bands were detected by Clarity western ECL substrate (BioRad) and visualized using a Chemidoc (BioRad). Densitometric analysis was performed using Image Lab software (BioRad) and individual Immunoreactive bands were normalized to immunoblots of actin levels for each sample.
Table 2.2: Antibodies used for protein detection

<table>
<thead>
<tr>
<th>Target Protein</th>
<th>Primary concentration</th>
<th>Secondary Concentration</th>
<th>Host</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin (1-19)</td>
<td>1:1000</td>
<td>1:10,000</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>BACE1</td>
<td>1:1000</td>
<td>1:10,000</td>
<td>Rabbit</td>
<td>Abcam</td>
</tr>
<tr>
<td>GGA3</td>
<td>1:1000</td>
<td>1:2500</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>APP</td>
<td>1:1000</td>
<td>1:2000</td>
<td>Mouse</td>
<td>Millipore</td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>1:1000</td>
<td>1:10,000</td>
<td>Mouse</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>ChAT C-terminal</td>
<td>1:1000</td>
<td>1:10,000</td>
<td>Rabbit</td>
<td>Immunoaffinity purified</td>
</tr>
<tr>
<td>ChAT N-terminal</td>
<td>1:250</td>
<td>1:5000</td>
<td>Rabbit</td>
<td>Immunoaffinity purified</td>
</tr>
<tr>
<td>Myc</td>
<td>1:1000</td>
<td>1:10,000</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Flag</td>
<td>1:1000</td>
<td>1:10,000</td>
<td>Rabbit</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

2.8 Immunofluorescence

To analyze the subcellular localization of 82-kDa ChAT, SH-SY5Y cells were fixed in freshly prepared 4% paraformaldehyde-PBS for 30 minutes at room temperature. Fixed cells were permeabilized with 0.5% Triton X-100 in PBS for 5 minutes, then blocked in PBS containing 3% normal goat serum for 30 minutes. SH-SY5Y cells were stained for ChAT by incubating with an immunoaffinity rabbit anti-ChAT antibody that recognizes 13 amino acids at the carboxyl-terminus of the protein (cTAB, 1:1000) overnight at 4°C. Cells were then incubated with an anti-rabbit secondary antibody conjugated to AlexaFluor 647 in blocking buffer (1:1000) for 1 hour at room temperature. Nuclei were stained with Hoechst dye (33342) in PBS (1:1000) for 5 minutes. Images were acquired with a Zeiss LSM 510 META laser-scanning confocal microscope using a 63X objective. Hoechst
staining was visualized using 405 nm excitation and a 420-480 nm emission, and 82-kDa ChAT was visualized with a 647 nm excitation and 650 nm emission using a long pass filter.

To visualize those neurons expressing 82-kDa ChAT following adenovirus transduction, primary cortical neurons were seeded on poly-L-ornithine coated confocal dishes (5 x 10^5 cells/dish) and grown for 6 days. Adenovirus was added 48 hours prior to processing for immunohistochemical analysis. Neurons were fixed in 4% paraformaldehyde-PBS for 30 minutes at room temperate, followed by blocking non-specific sites for 1 hour in PBS containing 0.05% Triton X-100 and 3% goat serum at room temperature. Neurons transduced with adenovirus to express 82-kDa ChAT or R560H 82-kDa ChAT were co-labeled for neuronal nuclear protein NeuN (mouse anti-NeuN antibody at 1:1000) and ChAT (rabbit cTAB antibody at 1:1000). Cells were then incubated with goat anti-rabbit secondary antibody conjugated to AlexaFluor 488 (1:500) and goat anti-mouse secondary antibody conjugated to AlexaFluor 555 (1:1000) in blocking buffer for 1 hour at room temperature. Images were acquired with a Zeiss LSM 510 META laser-scanning confocal microscope using a 63X objective. Immunofluorescence of 82-kDa ChAT and R560H 82-kDa ChAT was visualized by 488 nm excitation and 505-530 nm emission. NeuN immunofluorescence was visualized by 543 nm excitation and 560-615 nm emission.
2.9 Quantitative real-time PCR

Total RNA was isolated from PBS (pH 7.4)-washed cortical neuron cultures using the GeneElute kit (Sigma-Aldrich) at room temperature. RNA was reverse transcribed using the iScript cDNA synthesis kit (BioRad). qRT-PCR was carried out using the iQ SYBR Green Supermix (BioRad) on a BioRad CFX96 real-time thermocycler. Forward and reverse primers used were murine specific at a final concentration of 400 nM (Table 2.3). All genes were normalized to GAPDH and the fold change was calculated based on the ΔΔCt method relative to GFP-expressing cortical neurons. Statistical analysis was performed on normalized Ct values prior to transformation.

Table 2.3: Primers used for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>APP</td>
<td>Forward: AAGAACTTGCCCAAAGCTGA</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTCTTCTATTGGCTGCTTCC</td>
</tr>
<tr>
<td>GGA3</td>
<td>Forward: CAAAGGCTGAGCCCAAAGGT</td>
</tr>
<tr>
<td></td>
<td>Reverse: AAGGCACTGCTAGGCTTGAT</td>
</tr>
<tr>
<td>BACE1</td>
<td>Forward: TGGACTGCAAGGAGACGGAG</td>
</tr>
<tr>
<td></td>
<td>Reverse: AACAGTGCCCCGATGGATGACT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: TTGTGATGGGTGTAACCACGAG</td>
</tr>
<tr>
<td></td>
<td>Reverse: CATGAGCCCTTCCACAATGCCAAA</td>
</tr>
<tr>
<td>Sortilin 1</td>
<td>Forward: CCCGGACTTCATCGCCAAAG</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGGAGGAGAATAACCCCAAG</td>
</tr>
<tr>
<td>SNX6</td>
<td>Forward: TGCAGGTTACATTATCCCAACCA</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCCAGGTATTCAGCTTCCAGT</td>
</tr>
</tbody>
</table>
2.10 ChAT activity assay

Primary cortical neurons seeded on 35-mm dishes were transduced with either GFP, 82-kDa ChAT or R560H 82-kDa ChAT encoding adenovirus. ChAT activity was measured in total cell lysates using the radioenzymatic method of Fonnum (1969) as modified by Rylett et al. (1993).

2.11 BACE1 activity assay

BACE1 activity was measured in lysates of cortical neuron cultures using the β-secretase activity assay kit (BioVision), according to manufacturer’s instructions. Relative fluorescence units (RFU) was used as a measurement of BACE1 activity.

2.12 Human Aβ1-42 ELISA

Total levels of human Aβ1-42 were measured in media recovered from mouse cortical neuron cultures using the human Aβ1-42 ELISA kit (Invitrogen), according to the manufacturer’s protocols. All values were normalized to total protein concentrations of the cultures.

2.13 Luciferase promoter assay

Mouse SN56 cell were seeded at 30,000 cells in white 96-well plates and
co-transfected using Lipofectamine 2000 (Invitrogen) with 0.2 µg of one of the LightSwitch reporter GoClone plasmids (empty vector, R01, GAPDH or GGA3) and 0.2 µg of either 82-kDa ChAT, R560H 82-kDa ChAT, 69-kDa ChAT or pcDNA3.1 empty vector. Sequence alignment between the human GGA3 promoter construct and the GGA3 data base shows a 100% sequence match. R01 negative control reporter construct contains a 1kb non-conserved, non-genic, and non-repetitive fragment, while the GAPDH reporter construct served as a positive transfection control. Detection of promoter activation was mediated through the Renilla luciferase protein, which catalyzes oxidation of the coelenterazine substrate and produces light at 480 nm. Reporter assays were conducted 24 hours after transfection according to the LightSwitch luciferase assay system protocol (SwitchGear Genomics). Quadruplicate cell samples were used in all experiments.

2.14 Data Analysis

Statistical analysis was carried out on GraphPad Prism 5 using Student’s t-tests, or one-way ANOVA followed by Tukey’s, Dunnett’s or Bonferroni post-hoc test where appropriate. All data are presented as mean ± SEM with statistically significant differences determined as p ≤ 0.05. Each n value represents the number of independent experiments performed.
CHAPTER THREE: RESULTS

3.1 Genotyping of APP/PS1 transgenic mouse litters

The experimental model that was used in most of the studies reported in this thesis was cortical neurons cultured from the brains of APP/PS1 double transgenic mouse embryos. These cultures allowed us to test hypotheses using non-transformed neurons and to assess the effects of experimental manipulations on the production of Aβ peptides that are released from these neurons. These cultures also provided a control condition in each experiment for comparison with the transgenic neurons since some of the mouse embryos in each litter were non-transgenic mice. Tail clippings, taken from each embryo at 15 to 16 days of gestation, were digested and the genomic DNA was extracted for genotyping, as described in the Methods section. As illustrated in the example given in Figure 3.1, the genotyping PCR amplification reaction contained primers specific for each of the two transgenes, mutant human APP and PS1, which resulted in the production of amplicons of 350 bp and 608 bp in size respectively, and to endogenous mouse FLOXG which led to the production of a 171 bp amplicon as an internal control. Extraction buffer mixed with the PCR reaction buffer was used as a negative control, as indicated in the (−) lane (Figure 3.1)
Figure 3.1: Genotyping from APP/PS1 transgenic embryos. A representative genotyping from one litter that had 8 embryos following breeding of a wild-type female and double transgenic male mouse. Endogenous FLOXG (171 bp) was used as a PCR control. Transgenic embryos were confirmed by the presence of the APP and PS1 transgenes at 350 and 608 bp, respectively.
3.2 Expression of adenovirus in primary cortical neurons

Expression of GFP, 82-kDa ChAT or catalytically inactive R560H 82-kDa ChAT in primary cortical neurons was carried out using the adenoviral gene delivery system. Images were taken 8 days after isolation, following a 48 hr exposure of adenovirus. Figure 3.2 confirms that both the 82-kDa ChAT (Panel B) and R560H 82-kDa ChAT (Panel C) are expressed in the nuclei of primary cortical neurons at varying levels following adenoviral infection. Between 20 and 30% of neurons express wild-type or mutant ChAT at an MOI of 100. Cortical neurons transduced with a GFP adenovirus were used as a control for this study (Panel C). Further, 82-kDa ChAT is catalytically active in cortical neurons (93.85 nmol/mg/hr), where R560H 82-kDa ChAT lacks enzymatic activity (1.95 nmol/mg/hr) similar to GFP control (2.83 nmol/mg/hr) (Panel D).

3.3 Aβ_{1-42} release into culture media from APP/PS1 cortical neurons

Cortical neurons cultured from APP/PS1 transgenic mouse embryos provide a valuable model for studying the regulation of and the effects of experimental manipulations on the cellular metabolism of APP. To determine the levels of human Aβ_{1-42} produced by and released from these neurons, a sensitive ELISA was used to measure the levels present in the culture media. Figure 3.3 (Panel A) depicts a time-course of human Aβ_{1-42} levels in cultured media from both transgenic (TG) and non-transgenic (NTG) mice at 4, 8, and 11 DIV. A small increase in the amount of Aβ_{1-42} was produced in the media from the TG
Figure 3.2. Expression of adenovirus in primary cortical neurons. Primary cortical neurons transduced for 48 h with GFP (Panel A), 82-kDa ChAT (Panel B), or R560H 82-kDa ChAT (Panel C) adenovirus at an MOI of 100. 20 to 30% of cortical neurons counterstained with NeuN, express GFP or ChAT. For ChAT activity, data are expressed as mean of 2 independent experiments (Panel D).
Figure 3.3. Human Aβ₁₋₄₂ is released from APP/PS1 cortical neurons and is reduced by 82-kDa ChAT expression. Panel A shows human Aβ₁₋₄₂ levels measured from cultured media of NTG and APP/PS1 TG cortical neurons by ELISA. TG neurons had a significant increase in Aβ₁₋₄₂ release after 8 DIV compared to NTG littermates (n=5). ***p<0.0001, Two-way ANOVA, Bonferroni post-test. Expression of 82-kDa ChAT resulted in a significant reduction in soluble Aβ₁₋₄₂ levels 8 DIV compared to control (Panel B). R560H 82-kDa ChAT expression also reduced Aβ₁₋₄₂ levels, but this was not significant compared to control. Aβ₁₋₄₂ release was normalized to total protein concentration in cell lysates (n=5). *p<0.05, One-way ANOVA with repeated measures, Tukey post-test (Fatima Abji).
cultures at 4 DIV when compared to the NTG cultures (16.7 ± 7.8 versus 7.4 ± 3.2 pg/mL), but this was not found to be statistically significant. However at 8 DIV, there was a statistically significant increase in Aβ1-42 levels in the media recovered from the culture of TG cortical neurons when compared to the NTG cultures (284.8 ± 41.3 versus 5.2 ± 0.7 pg/mL). The level of Aβ1-42 continued to rise in the media from the TG cultures at 11 DIV (909 ± 28.2 versus 6.5 ± 0.3 pg/mL), but as expected this was not seen in the NTG cultures.

Importantly, when either the wild-type human 82-kDa ChAT protein or the catalytically-inactive mutant R560H 82-kDa ChAT protein was expressed in the cortical neurons cultured from the TG mice there was a reduction in the amount of human Aβ1-42 released into the culture media (Figure 3.3.B). Neurons were transduced with adenovirus encoding either GFP, 82-kDa ChAT or R560H 82-kDa ChAT and maintained in culture for 8 days, then culture media was collected and the levels of Aβ1-42 measured by ELISA and normalized to the total protein content in the cultures. Figure 3.3B displays a significant reduction in the amount of Aβ1-42 in media from cultures expressing wild-type 82-kDa ChAT (3.13 ± 0.68) compared to GFP-expressing control cultures (3.79 ± 0.83 pg/mL per µg protein) (P < 0.05, One-way ANOVA with repeated measures). R560H 82-kDa ChAT had a reduction in Aβ1-42 released in the media, but not significantly different from GFP control (3.18 ± 0.69 pg/mL per µg protein).
3.4 82-kDa ChAT alters mRNA and protein levels for APP processing-related genes

Primary cortical neurons prepared from APP/PS1 transgenic mouse embryos were virally-transduced to express either GFP, 82-kDa ChAT or the catalytically-inactive R560H 82-kDa ChAT mutant. Importantly, real-time PCR (qRT-PCR) analysis reveals a significant upregulation of GGA3 mRNA in neurons expressing either 82-kDa ChAT (1.42 ± 0.11) or R560H 82-kDa ChAT (1.57 ± 0.22) compared to GFP-expressing control neurons (Figure 3.4A). The steady-state levels of either APP or BACE1 mRNA were not significantly altered in neurons expressing either 82-kDa ChAT or R560H 82-kDa ChAT (Figure 3.4A).

Protein levels for GGA3, APP and BACE1 were measured by immunoblot analysis with Immunoreactive band intensities quantified by densitometry and normalized to sample actin immunoreactivity. Consistent with the changes observed for GGA3 mRNA, primary cortical neurons expressing either 82-kDa ChAT or R560H 82-kDa ChAT had a significant increase in the steady-state amount of GGA3 protein (1.55 ± 0.23 and 1.51 ± 0.25, respectively) compared to GFP-expressing control neurons (0.92 ± 0.22). In sharp contrast to the findings for GGA3 protein, there was a significant reduction in BACE1 protein levels in neurons expressing either 82-kDa ChAT (0.93 ± 0.15) or R560H 82-kDa ChAT (1.3 ± 0.31) when compared to GFP-expressing control neurons (1.84 ± 0.26). APP levels remained unchanged in neurons expressing both 82-kDa ChAT (1.38
Figure 3.4: Expression of 82-kDa ChAT and R560H 82-kDa ChAT alter BACE1 and BACE1 trafficking protein levels in APP/PS1 cortical neurons. mRNA (Panel A) and protein (Panel B) levels of APP, GGA3 and BACE1 were measured in APP/PS1 primary cortical neurons transduced with adenovirus for 48 h at an MOI of 100. The BACE1 trafficking mediator gene, GGA3, is upregulated in response to expression of both 82-kDa ChAT and R560H 82-kDa ChAT. One-way ANOVA with Tukey’s test *p<0.05 (n=13). Protein levels of APP, BACE1, and GGA3 were evaluated by immunoblot and showed an upregulation of GGA3 following ectopic expression of 82-kDa ChAT and R560H 82-kDa ChAT. Conversely, BACE1 protein levels were significantly reduced in these samples. *p<0.05, One-way ANOVA with Dunnett’s test (n=7). Panel C shows a significant reduction in BACE1 activity measured by a fluorescence assay in cortical neurons expressing both 82-kDa ChAT and R560H 82-kDa ChAT. **p<0.01, *p<0.05, One-way ANOVA with Tukey’s test (n=5). Data has been transformed and normalized to control, with raw values reported in the text.
± 0.58) or R560H 82-kDa ChAT (1.16 ± 0.27) compared to GFP control (1.23 ± 0.55) (Figure 3.4B). BACE1 activity was also measured in primary cortical neurons, and consistent with the findings of a reduction of BACE1 protein levels, the enzyme activity was also significantly reduced in neurons expressing either 82-kDa ChAT (1382 ± 357.5 RFU/µg protein, p ≤ 0.01) or R560H 82-kDa ChAT-expressing cells (1757 ± 412.5 RFU/µg protein, p ≤ 0.05) when compared to the GFP-expressing control neurons (2421 ± 568.4 RFU/µg protein) (Figure 3.4C).

### 3.5 82-kDa ChAT does not alter expression of all protein trafficking genes

The trafficking of BACE1 to lysosomes in conjunction with the actions of GGA3 plays an important role in maintaining its cellular levels and mediates its degradation (Tesco et al., 2007). Previous studies have further linked both SNX6 as a negative modulator of BACE1 transport (Okada et al., 2010) and Sortilin 1 as a retrograde transporter of BACE1 from the endosomes to the trans-Golgi network (TGN) (Finan et al., 2011). To determine whether the expression of 82-kDa ChAT protein in neurons has a generalized effect on proteins involved in trafficking, we investigated the effect of expressing either 82-kDa ChAT or R560H 82-kDa ChAT on two other trafficking proteins, SNX6 and SORT1, at the mRNA level. Figure 3.5 reveals that neither 82-kDa ChAT nor R560H 82-kDa ChAT significantly altered steady-state SNX6 or SORT1 transcript levels in primary cortical neurons when compared to GFP-expressing control neurons.
Figure 3.5: The trafficking genes, *SORT1* and *SNX6* were unchanged in response to overexpression of both 82-kDa ChAT and R560H 82-kDa ChAT in cortical cultures form APP/PS1 mice.
3.6 Chloroquine treatment restores BACE1 protein levels in ChAT expressing neurons

GGA3 has been shown to interact directly with ubiquitinated BACE1 and target it to the lysosome for degradation (Kang et al., 2010). To determine if the reduction in BACE1 levels observed in our studies in 82-kDa ChAT expressing neurons was the result of lysosomal degradation of the protein, APP/PS1 primary cortical neurons were treated with chloroquine, which deacidify lysosomes and reduces the activity of many lysosomal enzymes. In these experiments, APP/PS1 cultured neurons were transduced with adenovirus encoding either 82-kDa ChAT, R560H 82-kDa ChAT or GFP on day six in culture, then treated with 25 µM chloroquine for 18 hours prior to being harvested and lysates prepared for immunoblots for BACE1 protein analysis. As illustrated in Figure 3.6, cultured cortical neurons expressing either 82-kDa ChAT (0.96 ± 0.05) or R560H 82-kDa ChAT (0.93 ± 0.08) and treated with vehicle had a statistically significant reduction in BACE1 protein levels when compared to GFP-expressing control cells (1.25 ± 0.05). By comparison, chloroquine treatment of neurons expressing either 82-kDa ChAT or R560H 82-kDa ChAT attenuated the reduction in BACE1 protein levels, and restored BACE1 protein to control levels (1.23 ± 0.09 and 1.38 ± 0.08 respectively). There was a small increase in BACE1 levels in control neurons expressing GFP with chloroquine treatment (1.37 ± 0.07) (Figure 3.6).
Figure 3.6: Lysosomal inhibition restores BACE1 protein levels. Inhibition of lysosome function using 25µM Chloroquine for 18 hours results in attenuation of the effect of expressing 82-kDa ChAT in cultured neurons as demonstrated by an increase in BACE1 protein levels. *p<0.05, one-way ANOVA (n=7). Data has been transformed and normalized to control, with raw values reported in the text.
3.7 GGA3 knockdown restores BACE1 protein levels in 82-kDa ChAT expressing neurons

To assess further if the reduction of BACE1 protein levels and activity observed in cultured neurons expressing 82-kDa ChAT is mediated by a mechanism that involves GGA3, cortical neurons were treated with lentivirus that encodes an shRNA targeting GGA3 transcripts with no known cross-reactivity based on sequence analysis. Control neurons were transduced with a lentivirus encoding GFP (copGFP lentivirus). Figure 3.7A shows that the GGA3 protein levels were significantly increased in cultured neurons expressing 82-kDa ChAT when compared to GFP-expressing neurons (1.82 ± 0.35 and 1.20 ± 0.17, respectively. #p<0.05); in this experiment, both the 82-kDa ChAT and GFP expressing neurons were transduced with the copGFP lentivirus control virus. As predicted, neurons transduced with the GGA3 shRNA lentivirus had significant reductions in endogenous GGA3 protein levels to about 50% of control (Figure 3.7A). Figure 3.7B shows the effect of GGA3 knockdown on BACE1 protein levels. Consistent with our previous results (Figure 3.4B), in cultured cortical neurons treated with the control lentivirus copGFP, expression of 82-kDa ChAT resulted in a significant reduction in BACE1 protein level when compared to GFP-expressing control neurons (0.97 ± 0.12 and 1.39 ± 0.03, respectively). Importantly, the reduction in GGA3 protein levels caused by knockdown with the shRNA restored BACE1 protein levels in 82-kDa ChAT expressing neurons to the level of that found in GFP-expressing control neurons. Furthermore, BACE1 activity was measured and its activity in 82-kDa ChAT expressing neurons was
Figure 3.7: GGA3 knockdown recovers BACE1 protein levels in 82-kDa ChAT expressing neurons. Primary cortical neurons infected with 82-kDa ChAT and a copGFP control lentivirus had a significant increase in GGA3 expression (#p<0.05). Transfection of lentiviral shRNA targeting GGA3 resulted in a 50% reduction in expression levels (A). The reduction in GGA3 levels resulted in attenuation of the effect of expressing 82-kDa ChAT in cultured neurons as demonstrated by an increase in BACE1 protein levels (B) *p<0.05, one-way ANOVA (n=7). BACE1 activity was also recovered close to control levels following GGA3 shRNA treatment (C) *p<0.05, one-way ANOVA (n=10). Data has been transformed and normalized to control, with raw values reported in the text.
recovered to close to control levels following GGA3 knockdown (Figure 3.7C).

3.8 82-kDa ChAT activates the GGA3 promoter

The next set of experiments was designed to determine whether 82-kDa ChAT plays a role in regulating GGA3 gene expression. To accomplish this, we obtained a promoter construct containing about 1 kilobase of the proximal promoter of the human GGA3 gene driving a luciferase reporter gene (SwitchGear Genomics). Both negative (RO1) and positive (GAPDH) control luciferase promoter constructs were included in these experiments. These control constructs and the GGA3 promoter construct were transfected individually into SN56 cells. Cells were co-transfected with plasmids encoding either wild-type 82-kDa ChAT, R560H 82-kDa ChAT, 69-kDa ChAT, or empty pcDNA3.1 vector as a control, then the effect on promoter activation was determined by measuring luciferase activity. Figure 3.8A shows that the expression of either 82-kDa ChAT, R560H 82-kDa ChAT or 69-kDa ChAT resulted in a statistically significant increase in the GGA3 promoter-luciferase activity (33789 ± 3217, 32526 ± 2150, and 31837 ± 3525, respectively) when compared to the empty vector control cells (23153 ± 2461). Expression of 82-kDa ChAT, R560H 82-kDa ChAT and 69-kDa ChAT was measured and luciferase activity relative to protein expression was assessed. 69-kDa expresses at a higher level relative to both 82-kDa ChAT and
Figure 3.8. SN56 cells expressing 82-kDa ChAT, R560H 82-kDa ChAT and 69-kDa ChAT were able to induce luciferase activity through the GGA3 promoter. (A) Expression of 82-kDA ChAT, R560H 82-kDa ChAT and 69-kDa ChAT resulted in a significant increase in luciferase activity through the GGA3 promoter compared to empty vector control. *p<0.05, one-way ANOVA (n=4). (B) Both 82-kDa ChAT and R560H 82-kDa ChAT display a 2-fold increase in GGA3 promoter activation compared to 69-kDa ChAT based on their relative expression levels. (C) SN56 cells transfected with empty vector control displayed no ChAT expression (Lane 3).
R560H 82-kDa ChAT, which may account for the high level of GGA3 promoter activation (Figure 3.8C). Transformation of luciferase activity relative to protein expression resulted in a 2-fold increase in GGA3 promoter activation through 82-kDa ChAT and R560H 82-kDa Chat compared to 69-kDa ChAT (Figure 3.8B).

### 3.9 APP processing is not altered in cell lines

The effect of 82-kDa ChAT expression on APP processing was also examined in various neural cell lines, including SH-SY5Y, N2A and SN56 cells (Figure 3.9). SH-SY5Y cells ectopically expressing 82-kDa ChAT did not show either an increase in GGA3 protein levels or a reduction in BACE1 protein levels that was observed in cultured mouse brain cortical neurons. Endogenous levels of BACE1 protein were found to be quite low in SH-SY5Y cells when compared to N2A and SN56 cells (Figure 3.9A). N2A cells ectopically expressing 82-kDa ChAT displayed no increase in GGA3 protein levels or a reduction in BACE1 protein, similar to the SH-SY5Y cells (Figure 3.9B). In contrast, SN56 cells expressing 82-kDa ChAT show a small increase in GGA3 protein levels, but BACE1 levels remain unchanged compared to control cells (Figure 3.9C).
Figure 3.9. Expression of 82-kDa ChAT in multiple cell lines does not alter BACE1 or BACE1 trafficking protein levels. SH-SY5Y (A) and N2A (B) cells transfected with 82-kDa ChAT cDNA expression vector show no increase in GGA3 protein levels or a subsequent reduction in BACE1 protein levels compared to cells transfected with empty vector control. (C) SN56 cells have a small increase in GGA3 protein levels when expressing 82-kDa ChAT, but no change in BACE1 protein levels.
3.10 Oxidative stress induces alterations in ChAT structure and localization

ROS levels are elevated in AD in response to soluble Aβ levels (Huang et al., 1999), and they can have detrimental effects on protein structure and function, potentially through the formation of disulfide bonds at Cys residues. Given that ChAT has a high number of solvent exposed Cys residues, it was asked if ROS mediated oxidation of Cys residues has an effect on ChAT structure, function and subcellular localization. HEK 293 cells expressing myc-tagged 69-kDa ChAT or FLAG-tagged 82-kDa ChAT were treated with increasing concentrations of the oxidative agent hydrogen peroxide (H$_2$O$_2$). It was shown that there was a concentration dependent effect on higher molecular weight (HMW) structure formation under non-reducing conditions of 69-kDa ChAT (Figure 3.10) and an increase in 82-kDa ChAT HMW structures at higher concentrations of H$_2$O$_2$ (Figure 3.11). Further, this effect was reversed with the addition of reducing agents dithiothreitol and 2-mercaptoethanol that break disulfide bonds.

Many proteins, including GAPDH have altered subcellular localization in response to oxidative stress and disulfide formation (Cumming and Schubert, 2005). It has been also found that 82-kDa ChAT exhibits decreased nuclear localization in patients with MCI and AD, through mechanisms currently unknown (Gill et al., 2006). Here we investigated the effect of Aβ$_{1-42}$ treatment on cytoplasmic 82-kDa ChAT localization following a 5-day treatment on stable SH-SY5Y cells. A concentration dependent increase in cytoplasmic 82-kDa ChAT
Figure 3.10. Oxidative stress alters 69-kDa ChAT structure. Under non-reducing conditions, 69-kDa ChAT forms concentration dependent higher molecular weight structures following H$_2$O$_2$ treatment. These structures are lost with the addition of reducing agents.
Figure 3.11. **Oxidative stress alters 82-kDa ChAT structure.** Under non-reducing conditions, 82-kDa ChAT forms higher molecular weight structures at increasing concentrations of $\text{H}_2\text{O}_2$. These structures are lost with the addition of reducing agents.
Figure 3.12. Aβ1-42 induces changes 82-kDa ChAT localization. Stable expressing 82-kDa ChAT SH-SY5Y cells treated with Aβ1-42 for 5 days display a concentration dependent change in ChAT localization. Under vehicle treatment, 82-kDa ChAT is localized in the nucleus. Increasing the concentration to 500 nM, ChAT is observed to change localization to the cytoplasm of SH-SY5Y cells.
staining was observed in the 100 and 500 nM Aβ_{1-42} treated SH-SY5Y clonal cells compared with vehicle control (Figure 3.12).
CHAPTER 4: DISCUSSION

4.1 Significant findings

The goal of this study was to further investigate the link between the expression of 82-kDa ChAT in neurons and changes in the expression of genes involved in the amyloidogenic processing of APP. Previous studies from our laboratory demonstrated that ectopic expression of 82-kDa ChAT in cortical neurons derived from embryonic double transgenic mice that express mutant human forms of both APP and PS1 led to a reduction in the amount of Aβ that is released from the neurons into the culture media. In addition, we were interested in investigating the effect of acute exposure of neurons to Aβ on 82-kDa ChAT structure and localization; Aβ can lead to increased ROS production in cells and oxidation of cellular constituents including proteins.

Here we demonstrate that increased expression of human nuclear 82-kDa ChAT and catalytically-inactive R560H 82-kDa ChAT alter APP processing genes that promote a reduction in BACE1 activity and expression resulting in reduced Aβ production. Expression of both ChAT proteins in primary neurons resulted in upregulation of both the GGA3 transcript and protein levels, while BACE1 protein levels were reduced. Inhibition of the lysosome with chloroquine treatment and knockdown of GGA3 through lentiviral shRNA, was found to recover BACE1 protein levels back to control levels. GGA3 promoter analysis through a luciferase assay indicates that both 82-kDa ChAT and R560H 82-kDa ChAT are
able to directly or indirectly activate transcription of the GGA3 1037 bp promoter sequence.

We were also able to demonstrate a direct oxidative effect on both 69- and 82-kDa ChAT structure, as well as a localization change of 82-kDa ChAT following Aβ_{1-42} treatment. HEK293 cells expressing 69- or 82-kDa ChAT, when treated with increasing concentrations of H_{2}O_{2}, show a concentration dependent increase in higher molecular weight ChAT species under non-reducing conditions. Addition of a reducing agent that can break disulfide bonds, eliminated the high molecular forms of ChAT. Finally, 5-day treatment of SH-SY5Y cells stably expressing 82-kDa ChAT with Aβ_{1-42} showed a concentration dependent localization change, favoring cytoplasmic staining at higher levels of Aβ_{1-42}.

4.2 Contribution to current knowledge of 82-kDa ChAT

The role and function of 69-kDa ChAT as an ACh synthesizing enzyme in cytoplasm of cholinergic neurons has been studied widely. The significance of a nuclear isoform of ChAT acting as a potential regulator of gene expression, however, has become a topic of study only recently. Matsuo et al. (2010) demonstrated recently that 82-kDa ChAT can act as an activator of transcription, specifically of the high-affinity choline transporter (CHT1). Previous studies in our laboratory indicate a possible role for ChAT in reducing both BACE1 protein levels and Aβ secretion (unpublished data). The current studies provide
additional support for a nuclear function of ChAT, in which it interferes with the amyloidogenic pathway through upregulation of the GGA3 protein. GGA3 has been linked to the APP processing pathway as a negative regulator of BACE1 through a direct interaction in which GGA3 binds to and targets ubiquitinated BACE1 to the lysosome for degradation (Kang et al., 2010). Here, we demonstrate that both wild-type 82-kDa ChAT and the catalytically-inactive mutant R560H 82-kDa ChAT regulate GGA3 at the level of transcription and this is mediated through an action at the level of the GGA3 promoter. It is still unknown whether the activation is via a direct or indirect mechanism. There is preliminary evidence that 82-kDa ChAT can bind directly to DNA. Our laboratory is currently investigating the ability of 82-kDa ChAT to bind directly DNA using both confocal microscopy and ChIP analysis. Analysis of the three-dimensional structure of ChAT shows a row of basic amino acid residues that lie along the surface of the protein and may be able to bind directly to the minor groove of DNA, at A-T rich regions. In preliminary studies, cells immunostained for 82-kDa ChAT show localization around DNA that is displaced by increasing concentrations of Hoechst (33342) dye, a nuclear stain that has the ability to displace proteins bound to the minor groove of DNA.

In the current study, we found that expression of a catalytically-inactive mutant of 82-kDa ChAT in neurons had the same affect as expression of the wild-type enzyme in all of the assays performed. This differs from the findings of Matsuo and colleagues (2010) in that they used a catalytically-inactive mutant of ChAT and obtained results that differed from those obtained with the wild-type
enzyme. In their study, Matsuo et al. (2010) did not find activation of the CHT1 gene in cells expressing inactive 82-kDa ChAT, whereas we demonstrate that catalytically-inactive 82-kDa ChAT maintains its ability to increase GGA3 levels. While these studies focused on the activation of two separate genes, it is also important to consider whether there may be potential differences between the two different mutant ChAT proteins expressed. We created a catalytically-inactive ChAT protein by mutating arginine 560 to histidine (R560H in 82-kDa ChAT, R442H in 69-kDa ChAT), which was reported as a spontaneously occurring mutant form of ChAT caused by a single nucleotide polymorphism (SNP) in individuals with congenital myasthenic syndrome that leads to apnea, paralysis, and death (Ohno et al. 2001; Dobransky et al. 2003). This mutation is found adjacent to the catalytic domain of ChAT, and it severely interrupts the activity of the enzyme by decreasing the affinity for binding of the co-substrate acetyl-CoA (Ohno et al., 2001). Moreover, our laboratory demonstrated that this mutant does not undergo protein kinase C-mediated phosphorylation of serine-440 which is also required for maintenance of optimal enzyme catalytic activity (Dobransky et al., 2004). Matsuo et al. (2010) created a mutant ChAT protein in which glutamic acid at residue 441 was substituted by a lysine residue (E441K). While this enzyme was catalytically-inactive, it also is expressed at a much lower level than either wild-type 82-kDa ChAT or the mutant R560H 82-kDa ChAT (Ohno et al. 2001). Interestingly, the study published by Matsuo et al. (2010) shows that this mutant is expressed at a low level, but they do not comment on how this may have affected their results. While it is unclear if glutamic acid 441 is
necessary for ChATs genetic regulation, either the stability or expression of this form of 82-kDa ChAT may account for the lack of effect on CHT1 expression.

We investigated the link between the expression of 82-kDa ChAT in neurons and the modulation of the GGA3-mediated BACE1 trafficking pathway further by interfering with other potential components of the system. We first focused on the effects of inhibiting the function of the lysosome through chloroquine treatment, which prevents endosomal acidification and decreases protein degradation. This in turn inhibits the function of lysosomal proteins and the fusion of endosomes with lysosomes, which require an acidic environment (De Duve, 1983). Chloroquine treatment has been previously shown to disrupt the lysosomal protein degradation pathway and elevate endogenous BACE1 levels in multiple cell types, including primary neurons (Koh et al., 2005). Here we demonstrate that treating cortical neurons cultured from APP/PS1 transgenic mice that express either 82-kDa ChAT or R560H 82-kDa ChAT with chloroquine can restore BACE1 protein levels to those observed in control neurons that do not express 82-kDa ChAT. Previous studies that investigated the mechanisms by which BACE1 protein is degraded provide conflicting results. One study shows that BACE1 is turned over through the ubiquitin-proteasome pathway, although these investigators did not test lysosomal inhibitors on BACE degradation as a control (Qing et al., 2004). These findings were challenged by Koh et al. (2005) who found that lysosomal inhibitors and not proteasome inhibitors cause an elevation in BACE1 protein levels, therefore suggesting that BACE1 is degraded in the lysosome.
It has been shown that experimental conditions that lead to a decrease in the level of GGA3 protein can result in elevation of BACE1 protein levels (Tesco et al., 2007). Samples from the temporal cortex of brains of individuals diagnosed with AD show a reduction in GGA3 protein levels that correlate inversely with increased BACE1 levels (Tesco et al., 2007). In the present studies, we demonstrate that shRNA-mediated reduction of GGA3 protein levels in cultured cortical neurons expressing 82-kDa ChAT results in elevated BACE1 protein levels similar to those observed in control neurons not expressing 82-kDa ChAT. GGA3 regulates BACE1 trafficking between the cell membrane and the TGN, as well as its transport to the lysosome for degradation mediated through a direct interaction of GGA3 with ubiquitinated BACE1. Depletion of cellular GGA3 protein interrupts the trafficking to both the lysosome and the TGN causing an accumulation of BACE1 in the early endosomes. The acidic environment of the early endosomes results in increased β-secretase activity of BACE1, thereby promoting increased amyloidogenic cleavage of APP and production of Aβ (Kang et al., 2010). While we did not observe a significant increase in BACE1 activity following GGA3 protein knockdown, future studies should investigate if there are changes in BACE1 localization in early endosomes in this model. Finally, we investigated the effect of expression of 82-kDa ChAT in the cultured cortical neurons on the expression of two other trafficking genes that are known to interact directly with BACE1 to determine if the ChAT-induced effects on BACE1 trafficking are more generalized. Sortilin facilitates the retrograde transport of BACE1 from the endosome to the TGN and promotes the interaction of APP and
BACE1, thereby increasing the production of Aβ (Tan and Evin, 2012). SNX6 promotes the trafficking of BACE1 in a manner that perturbs the accumulation in the TGN and reduces the interaction between APP and BACE1 (Okada et al., 2010). In both instances, the expression of 82-kDa ChAT in neurons had no effect on the expression of the genes for these two other known BACE1 trafficking genes, indicating that the effect noted for GGA3 is a specific effect in terms of the trafficking proteins monitored.

We also found that the effects mediated by 82-kDa ChAT on the activation of expression of some genes is cell specific. This was also observed in the studies reported by Matsuo et al. (2010) where CHT1 activation was observed in neuronal cells expressing 82-kDa ChAT, but not in HEK293 cells. Here, we demonstrate a cortical neuron-specific effect of 82-kDa ChAT on the expression of GGA3, but there was no effect in transformed neural cell lines that were tested. The reasons for this cell-specific effect of 82-kDa ChAT are not known, but cortical neuron specific activation of selected genes may be in place to compensate for the enhanced APP processing that takes place in this transgenic mouse model of AD.

To summarize, our results demonstrate a cell-specific secondary function of ChAT by which nuclear 82-kDa ChAT can facilitate genetic regulation through an upregulation of GGA3 at the mRNA and protein levels. This translates to a reduction in BACE1 protein and activity levels, mediated through enhanced lysosomal degradation of BACE1, culminating in a reduction in Aβ generation and secretion. This is illustrated in the schematic shown in Figure 4.1.
Figure 4.1: Proposed mechanism of nuclear 82-kDa ChAT regulation of APP processing. 82-kDa ChAT expression causes upregulation of GGA3 mRNA and protein which functions to target BACE1 for lysosomal-mediated degradation and decreased amyloidogenic processing.
4.3 Contribution to the effect of oxidative stress on ChAT

Increased levels of oxidative stress in the human AD brain are well documented (Markesbery, 1996). A range of cellular changes show that oxidative stress precedes any of the hallmark characteristics of AD, including Aβ deposition and neurofibrillary tangle formation. Aβ, particularly toxic Aβ1-42, is involved both directly and indirectly in the generation of ROS, which can cause the oxidation of lipids and proteins (Butterfield and Bush, 2004; Lahiri and Greig, 2004). Modification of reactive Cys thiols occurs during oxidative stress and can result in either a loss-of-function or gain-of-function for many proteins (Klatt and Lamas, 2001). Importantly, human ChAT has a higher than usual content of Cys residues, with 20 and 24 Cys residues in the 69-kDa and 82-kDa enzymes respectively, making it particularly vulnerable to oxidative stress. Here we investigated the effect of oxidative stress, both directly (H₂O₂) and indirectly (Aβ1-42) on ChAT structure. Both 69- and 82-kDa ChAT were shown to have a concentration dependent increase in the abundance of higher molecular mass structure formation under non-reducing conditions. Addition of the reducing agents dithiothreitol and 2-mercaptoethanol, agents which promote breakage of disulfide bonds, eliminated the higher molecular mass ChAT structures. These results are suggestive of an oxidation-dependent change in ChAT protein structure that may be mediated through oxidation at reactive Cys residues.

A decline in ChAT function has been linked to oxidative stress-mediated mechanisms initiated by Aβ oligomers, with this effect on ChAT activity reversed through the addition of antioxidant agents (Nunes-Tavares et al., 2012). At low
nanomolar concentrations of Aβ oligomers, ChAT activity can be reduced up to 50%, but this occurs in the absence of any evidence of neuronal damage or death (Nunes-Tavares et al., 2012). Moreover, there is growing evidence linking the ability for Aβ oligomers to activate the NMDA receptor and an increase in intracellular ROS levels (De Felice et al., 2007). In association with this, it was found that blocking the binding of Aβ oligomers to NMDA receptors by the AD drug memantine significantly reduced cellular ROS levels (De Felice et al., 2007). Taken together, reduction in ChAT activity mediated by Aβ oligomer-induced oxidative stress may be an early event in the development of AD pathology. Further study should investigate whether the formation of higher molecular mass ChAT species directly results in the decline in ChAT activity.

It has been demonstrated that there is decreased amounts of immunoreactive 82-kDa ChAT protein in nuclei of cholinergic neurons in necropsy brain from individuals diagnosed with MCI or early AD, but the mechanisms underlying this are currently unknown (Gill et al., 2007). Cumming and Schubert (2005) demonstrated that long-term treatment of cultured cells with Aβ can cause an oxidation-dependent change in the subcellular localization of GAPDH. In these studies, it was shown that GAPDH becomes oxidized to form dimers through disulfide linkage, and then can be readily shuttled into the nucleus to become a proapoptotic factor (Cumming and Schubert, 2005). Importantly, in the studies reported in this thesis, we demonstrate that with acute exposure of neural cells to Aβ oligomers, 82-kDa ChAT may undergo oxidation. Moreover, ChAT oxidation is associated with an Aβ oligomer concentration-
dependent change in its subcellular localization, favoring a predominately cytoplasmic localization similar to that seen in older individuals and in MCI and AD patients.

In summary, further study needs to be carried out to determine the cellular mechanisms that regulate changes in the subcellular, cytoplasmic versus nuclear, localization of 82-kDa ChAT. If the changes in subcellular compartmentalization of 82-kDa ChAT is a result of dimerization or oligomerization of the protein mediated by oxidative stress, then determining if this may potentially involve other cellular interacting or binding proteins with 82-kDa ChAT may give a better insight into the localization change. Finally, we demonstrated here that 82-kDa ChAT can elicit an effect on gene expression while located in the nucleus. If long-term changes in the oxidation state in the brain microenvironment can cause changes in the subcellular localization of 82-kDa ChAT, then it should be determined if the protective effect of 82-kDa ChAT expression against amyloidogenic APP processing is lost as a result of increased cellular ROS levels and post-translational oxidation of ChAT.

4.4 Conclusions

To our knowledge, this is the first time 82-kDa ChAT has been directly linked to regulation of components of the amyloidogenic APP processing pathway. Nuclear 82-kDa ChAT is able to cause a reduction in both BACE1 protein and activity and in Aβ levels in cortical neurons from APP/PS1 double
transgenic mice, mediated through regulation of the GGA3 trafficking protein, and independent of ChAT enzymatic activity. Both the inhibition of lysosome function and knockdown of GGA3 protein levels were able to restore BACE1 protein levels to control levels in neurons expressing 82-kDa ChAT. Finally, short-term and long-term oxidative stress are able to alter ChAT structure and subcellular localization, respectively. Under normal cellular conditions, 82-kDa ChAT is localized to the nucleus of neurons and is able to regulate GGA3 gene expression and protein levels and cause a reduction in BACE1 protein levels, thereby reducing the level of Aβ secretion into media bathing cultured neurons expressing mutant human APP and PS1. It can be hypothesized that as AD pathology progresses and the level of cellular ROS increases, the subcellular localization of 82-kDa ChAT in cholinergic neurons changes to a predominately cytoplasmic localization. Further study should focus on the events that lead to either the loss of nuclear import of 82-kDa ChAT or increases in its nuclear export, and whether this ultimately results in a loss of its effects on the regulation of gene expression.

4.5 Limitations of the Research

The major limitation for the present study was achieving consistent expression of the GFP and ChAT adenovirus in neurons cultured from mouse brain cortex. Using an adenoviral gene delivery system was the most effective approach for these studies, but this system was only able to yield approximately
20-30% transduction efficiency at an MOI of 100. A lower MOI produced a reduced yield of expressing cells and a higher MOI became toxic to the neurons. During the knockdown experiments, neurons co-transfected with both a lentivirus and adenovirus had a reduced level of co-expression, which may account for the smaller changes observed for several measures and the greater variability in data, for example in the BACE1 activity assays. Finally, cultures with higher than normal astrocyte levels display lower neuronal expression of adenoviral expressed genes as a result of being taken up more readily by the astrocyte population. To overcome this, our lab is in the process of developing a transgenic mouse strain in which Floxed 82-kDa ChAT is expressed in forebrain cholinergic neurons and crossing with Cre-driver mice. This will remove the variable expression between cultures and provide a better model to study the effect of 82-kDa ChAT expression on the APP processing pathway.

The effect of long term exposure to Aβ on 82-kDa ChAT structure and subcellular localization in primary cortical neurons could not be carried out as a result of a limited expression period of the adenovirus. Expression of 82-kDa ChAT in primary neurons peaked between 2 and 3 days post-transfection, thereby limiting their ability to be used in experiments requiring a 5 day test period. Finally, the generation of a lentiviral delivery system for expression of 82-kDa ChAT or the creation of knock-in mice that express 82-kDa ChAT in neurons would allow for and extended time point analysis of Aβ-induced oxidative stress on 82-kDa ChAT localization in cultured neurons. Offspring of a 82-kDa ChAT X Cre mouse crossed with a APP/PS1 mouse could also give insight into the long-
term effect of 82-kDa ChAT expression in AD and to possible subcellular localization alterations resulting from an overactive amyloidogenic pathway.

4.6 Future Studies

The studies reported in this thesis demonstrate a novel role for both 82-kDa ChAT and R560H 82-kDa ChAT in regulation of the APP processing pathway. The present data indicates that nuclear localized ChAT is able to drive activation of the GGA3 promoter, resulting in increased levels of GGA3 mRNA and protein. Determining whether this effect of 82-kDa ChAT is mediated through a direct interaction with DNA or by an indirect interaction, possibly mediated through transcription factor cascade or other regulatory proteins, is an important next step.

Short term ROS treatment to cells expressing both 69- and 82-kDa ChAT showed a concentration-dependent increase in higher molecular mass ChAT structures. While some of the higher molecular mass structures coincide with a molecular weight that would suggest homo-dimerization, there are oxidized forms of ChAT that may have dimerized with other cellular proteins. Redox 2D SDS-page to separate possible proteins that form mixed disulfide bonds with ChAT as a result of oxidation may give insight into the cause of both a reduction in ChAT activity and localization change observed from both short- and long-term oxidative stress.
The generation of a transgenic knock-in model of 82-kDa ChAT will allow for further and more complete analysis of the role of this cholinergic neuronal enzyme in role in APP processing. A major limitation in the present studies was consistent adenovirus expression of ChAT proteins, which would be overcome by generation of knock-in mice model. Further, the effect of long-term Aβ exposure to neurons expressing 82-kDa ChAT could be examined using progeny of a knock-in mouse crossed with an APP/PS1 transgenic mouse.
CHAPTER 5: REFERENCES


Vassar R., Bennett BD., Babu-Khan S., Kahn S., Mendiaz EA., Denis P., Teplow DB., Ross S., Amarante P., Leoloff R., Luo Y., Fisher S., Fuller J., Edenson S.,


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London Health Research Day, March 2012

Nuclear choline acetyltransferase decreases amyloidogenic APP metabolism in neurons from APP/PS1 transgenic mice
Society for Neuroscience Annual Meeting, October 2012

Nuclear choline acetyltransferase decreases amyloidogenic APP metabolism in neurons from APP/PS1 transgenic mice
PhysPharm Research Day, November 2012

Nuclear choline acetyltransferase decreases amyloidogenic APP metabolism in neurons from APP/PS1 transgenic mice
London Health Research Day, March 2013
Publications: