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DONEPEZIL AND GALANIN INTERACTIONS IN AN ANIMAL MODEL OF ALZHEIMER'S DISEASE

by

Jonathan Jacob Sabbagh

Bachelor of Science
Tulane University
2004

A thesis submitted in partial fulfillment
of the requirements for the

Master of Arts in Psychology
Department of Psychology
College of Liberal Arts

Graduate College
University of Nevada, Las Vegas
December 2010

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THE GRADUATE COLLEGE

We recommend the thesis prepared under our supervision by

Jonathan Jacob Sabbagh

entitled

Donepezil and Galanin Interactions in an Animal Model of Alzheimer's Disease

be accepted in partial fulfillment of the requirements for the degree of

Master of Arts in Psychology

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December 2010

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ABSTRACT

Donepezil and Galanin Interactions in an Animal Model of Alzheimer's Disease

by

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Alzheimer's disease (AD) is a neurodegenerative disorder marked by a progressive loss of cognitive function. One of the neurobiological hallmarks of AD is a progressive loss of cholinergic neurons and a decrease in the amount of acetylcholine in the brain. Pharmacological therapies have targeted the cholinergic system, specifically first-line, palliative treatment using acetylcholinesterase (AChE) inhibitors, such as donepezil. Donepezil has been shown to increase cholinergic tone and ameliorate some of the cognitive deficits in AD patients. Galanin, a neuropeptide that inhibits the evoked release of several neurotransmitters including acetylcholine as well as modulates seveal intracellular cascades, is overexpressed in AD resulting in an as yet unidentified modulation of neurobiological function. Galanin also impairs learning and memory when administered centrally to rodents, suggesting it may contribute to the cognitive impairments observed in AD. While the mechanism by which galanin impairs learning has yet to be determined, studies suggest it is through cholinergic mechanisms. We investigated the ability of donepezil to rescue learning and memory deficits induced by galanin administration, and by extension isolated whether the learning impairments produced by galanin were ameliorated by increasing cholinergic tone. We also investigated the effects of donepezil and galanin in an animal model of AD, i.e. their

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effects on learning and memory following a slight lesion of cholinergic neurons analogous to the cholinergic loss seen in AD. This study provides vital information about the relationship between galanin-induced deficits and acetylcholine, and helps to clarify the roles of donepezil and galanin in AD.

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TABLE OF CONTENTS

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CHAPTER 1

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive loss of memory and a decline in cognitive function (Heese & Akatsu, 2006). It is the most common cause of age-related dementia accounting for 50-60% of age-related cases. The average age of onset for AD is 65 years, while most cases occurring before this age are referred to as early-onset familial AD, with identifiable genetic links. Other symptoms accompanying the memory loss are confusion, disorientation, anxiety, delusions and apathy or depression (Terry & Katzman, 1983). As the disease advances, symptoms may include anger, aggression, language problems, and impaired motor function (Souren et al., 1995; Waldemar et al., 2007). In addition to the behavioral disruptions associated with AD, several pathological changes have been observed in the brain including beta-amyloid (Aβ) plaque deposition, neurofibrillary tangle formation, and the progressive loss of cholinergic neurons (Bartus et al., 1982; Glenner & Wong, 1984; Masters et al., 1985; Arriagada et al., 1992; Goedert, 1996). These pathological changes are the hallmarks of the disease, and may be responsible for the cognitive and behavioral deficits exhibited in AD.

One of the most extensively investigated hallmarks of the disorder is the senile plaques observed in post mortem examinations. Aβ proteins form the core of senile plaques, one of the pathological changes which may be inducing the neuronal loss seen in AD (Glenner & Wong, 1984; Masters et al., 1985). Senile plaques are extracellular structures composed mainly of aggregated Aβ, and they are seen almost exclusively in AD and AD-related pathologies like Down syndrome (Wisniewski et al., 1985). Several

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studies suggest that the accumulation of Aβ in the brain may initiate or lead to the pathogenesis of AD (Selkoe, 2001). These findings and others have led to the amyloid cascade hypothesis (Hardy & Higgins, 1992), which suggests that the amyloid deposits that form plaques are the causative event in AD and the resulting neurodegeneration is a by-product of this buildup. Evidence supporting this hypothesis comes from genetic studies showing mutations in the genes associated with familial and late-onset AD lead to increased Aβ aggregation and cognitive deficits (Goate et al., 1991; Murrell et al., 1991; Corder et al., 1993; Levy-Lahad et al., 1995; Sherrington et al., 1995).

Investigators have been searching for genetic ties to AD for years in an attempt to determine the etiology of the disease but with only mild success. Although a few genes have been implicated as risk factors, none have yet provided a clear link between the pathogenesis of AD and specific genetic targets, with most promising targets tied to familial AD. Mutations in the amyloid precursor protein (APP), which is responsible for the formation of Aβ peptides and whose encoding gene is located on chromosome 21, have been linked to early-onset familial AD (Goate et al., 1991; Murrell et al., 1991). Also, patients with Down syndrome, a trisomy (additional copy) of chromosome 21, show AD pathology by 40 years of age (Holtzman et al., 1996). Alternative genetic approaches have implicated mutations in genes called presenilins (PS) which have also been tied to early-onset AD, specifically presenilin-1 (PS-1) and presenilin-2 (PS-2) located on chromosomes 14 and 1, respectively (Levy-Lahad et al., 1995; Sherrington et al., 1995). In AD, genetic investigations have implicated a specific allele of Apolipoprotein E (ApoE), whose gene is localized on chromosome 19, which has been linked to an increased likelihood of developing the disease (Corder et al., 1993).

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Although genetic linkage studies have provided useful insight into potential AD etiology, genetic mutations related to Aβ can only account for a small percentage of AD cases. A further limitation of the Aβ hypothesis is that many studies have indicated that there is little to no correlation between the number or size of amyloid deposits and the severity of the dementia, and that other pathologies seem to correlate better with the memory loss seen in AD (Terry et al., 1991; Arriagada et al., 1992). Therefore, additional pathologies such as the hyperphosphorylation of tau have been the focus of much research.

Neurofibrillary tangles (NFTs) are another neuropathological hallmark of AD and are composed mainly of hyperphosphorylated tau, a protein that is associated with microtubule stability and assembly. Tau hyperphosphorylation leads to the formation of paired helical filaments (PHF) which are thought to lead to microtubule disintegration and neuron death (Goedert, 1996). Because neurofibrillary pathology, as well as the number of cortical tangles, correlates positively with the severity of dementia in AD (Arriagada et al., 1992), it is of great interest to investigators of the disorder and those developing pharmacological treatments. In addition, the regions of the brain that appear to undergo the greatest degeneration of neurons and synapses in AD are those that project to or from areas that have high densities of plaques and tangles, specifically the hippocampus, neocortex, and basal forebrain (for review, see Kar et al., 2004). The latter region contains large numbers of cholinergic neurons which project to the hippocampus and cortex. Acetylcholine (ACh), principally an excitatory neurotransmitter, is important for attentional processes, as well as learning and memory (Deutsch, 1971; Wenk et al., 1994; Woolf, 1996). A reduction in neurons containing ACh has been consistently observed in AD, particularly in the early stages of the disorder.

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Cholinergic cell loss is a hallmark of the neurodegeneration in AD, marked by a progressive loss of ACh-containing neurons with a corresponding decline in cognitive function (Perry et al., 1981; Terry et al., 1991). The cholinergic hypothesis of AD postulates that the cognitive deficits in AD are caused by the early loss of cholinergic basal forebrain (CBF) neurons (Bartus et al., 1982). This hypothesis is supported by many studies which demonstrate that the loss of CBF neurons occurs early in the disease progression, likely before a clinical diagnosis is reached (Bartus et al., 1982; Bowen et al., 1982; Beach et al., 1997; Beach et al., 2000). Also, the severity of the dementia in AD is highly correlated with the amount of cholinergic loss (Perry et al., 1981). Furthermore, the first drugs to be developed for AD have targeted the cholinergic system with moderate effectiveness. Donepezil, the first FDA-approved drug for AD and also the most widely prescribed, has shown to be effective at ameliorating behavioral symptoms associated with learning and memory in the early stages of the disease (Rogers et al., 2000). Donepezil is an acetylcholinesterase (AChE) inhibitor, effectively reducing the extent of the breakdown of ACh and increasing the available amount of ACh at the synapse (Sugimoto et al., 1990). It has long been thought that AChE inhibitors only have the ability to alleviate the symptoms of AD, but recent studies suggest they may have disease-modifying effects as well (Sabbagh et al., 2006). One possible explanation as to why these CBF neurons are dying is related to calcium levels and excitotoxicity (Lucas & Newhouse, 1957).

Excitotoxicity results from the overload or overabundance of calcium ions in neurons, and can be caused by the excessive activation of several receptors including glutamate receptors (Lucas & Newhouse, 1957). This excessive receptor activation can

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lead to a large and persistent influx of calcium into the cell which produces too much excitation that can lead to cell death. Excitotoxicity has been tied to several neurodegenerative disorders including Huntington's disease, amyotrophic lateral sclerosis (ALS), and AD (Mattson et al., 1992; Taylor-Robinson et al., 1994; Cluskey & Ramsden, 2001). In AD specifically, increased calcium levels have been found in cells containing NFT and may even precede tangle formation (McKee et al., 1990). Furthermore, Aβ production is increased as a result of calcium influx *in vitro* (Querfurth & Selkoe, 1994). An endogenous neuropeptide that seems to be effective at reducing excitotoxicity and which has the capacity to protect neurons from overactivity (i.e. trophic properties) is galanin (Cortes et al., 1990).

Galanin acts primarily as a neuromodulator in the central nervous system (CNS), interacting with several neurotransmitters including ACh, serotonin (5-HT), glutamate, and norepinephrine (Dutar et al., 1989; Pieribone et al., 1995; Kinney et al., 1998; Xu et al., 1998). Galanin is also overexpressed in AD, hyperinnervating surviving CBF neurons at concentrations as high as twice that of age-matched controls (Chan-Palay, 1988; Beal et al., 1990). When administered into the lateral ventricles or hippocampus of rodents, galanin has been shown to impair reference and working memory in several learning and memory tasks, including the Morris water task, T-maze delayed alternation, delayed non-matching to position, starburst radial maze and trace fear conditioning (Mastropaolo et al., 1988; Robinson & Crawley, 1993; Ogren et al., 1996; Kinney et al., 2002; Kinney et al., 2003). Based on these findings, it is possible that galanin is contributing to the cognitive deficits observed in AD by inhibiting CBF neurons in an attempt to serve a neuroprotective role. One important question that has not been directly investigated is

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whether galanin specifically modulating cholinergic tone may be responsible for the learning impairments above, which would have large implications for galanin in AD.

Research Questions

The present study investigated in experiment 1 if donepezil, the aforementioned AChE inhibitor, was able to rescue any of the cognitive impairment induced by central galanin administration consistent with other investigations. Because galanin has been shown to inhibit cholinergic signaling (Fisone et al., 1987), donepezil may be able to reverse the galanin-induced deficit by increasing cholinergic tone. If donepezil is able to rescue the learning and memory deficits in the galanin-infused animals, then it suggests that galanin may produce a deficit by modulating cholinergic mechanisms. However, if donepezil is unable to significantly rescue the galanin-induced deficit, it is likely the learning and memory impairments produced by galanin are unrelated to its suppression of cholinergic activity. Further, these data would also provide important information regarding the galanin-induced deficit reported in other studies. Such findings would indicate that galanin's role in AD may not be as closely tied to the cholinergic system as the literature suggests. Either way, the first part of our study would provide crucial insight into galanin-ACh interactions, as well as perhaps answer some questions as to galanin's role in AD.

In experiment 2, we examined the effect that galanin and donepezil have on learning and memory, individually as well as in combination, following administration of the cholinergic neurotoxin, ethylcholine mustard aziridinium (AF64A). AF64A is a selective cytotoxin which specifically targets cholinergic neurons and produces

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behavioral and histological deficits in the targeted region (for review see Smith, 1988). The effects of a cholinergic lesion on spatial learning and memory have been quite inconsistent with many studies finding a deficit while others have not (Walsh et al., 1984; Lamberty et al., 1992; Nakamura et al., 1992; Opello et al., 1993; Dornan et al., 1996; Bizon et al., 2003; Frick et al., 2004; Dashniani et al., 2009). The extent of the lesion and the amount of toxin administered likely influence whether or not deficits are observed following cholinergic-specific lesions. Our study utilized a relatively low dose of AF64A in order to damage but not fully destroy the cholinergic system. Further, our intent was to mimic the cholinergic loss observed in early or even pre-clinical AD necessitating a smaller dose of the toxin.

We hypothesized that co-administration of donepezil and galanin following an AF64A-induced cholinergic lesion would in part rescue any learning and memory deficits caused by the toxin and potentially preserve a large number of cholinergic neurons. It may be possible that following the cholinergic lesion, galanin may preserve some cholinergic function, if it is in fact a trophic factor *in vivo*, and donepezil may behaviorally offset the AF64A-induced loss of ACh. If donepezil and galanin together are able to ameliorate the cognitive impairment and cell loss to a greater degree than just donepezil alone, then this finding would lend significant support to the argument for developing galanin agonists as an adjunctive treatment with donepezil in AD patients. Alternatively, if donepezil and galanin administration fails to improve performance or cholinergic neuron survival, this finding would suggest the utility of galanin antagonists in AD (if the increase in survival is absent, galanin may simply be exacerbating the cognitive impairment). Regardless of the findings, based on the overlap of the efficacy of

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donepezil to ameliorate AD symptoms and the overexpression of galanin in AD, this investigation will contribute to the understanding of each of their roles in the disorder. We are genuinely surprised that there is no literature along these lines and this experiment should reveal insightful information about AD and the possible ways it can be therapeutically treated in the future. Below we have outlined a more comprehensive review of each of the aforementioned approaches and findings in AD.

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CHAPTER 2

REVIEW OF RELATED LITERATURE

Amyloid β Hypothesis

The Aβ protein accumulates extracellularly in AD resulting in the formation of senile plaques, which may lead to cell damage and even cell loss. Aβ was first purified in 1984 from cerebrovascular amyloid protein by Glenner & Wong and from senile plaques the next year by Masters et al. (1985), which was when its amino acid sequence was determined. Various mechanisms have been suggested to account for the neurotoxicity of Aβ peptides and senile plaques. Various studies have discovered a disruption in calcium homeostasis following Aβ administration which could potentially lead to excitotoxicity and cell loss. Further, it has been suggested that plaques may disturb surrounding cytoskeletal elements by "squishing" nearby cells. Although the mechanism by which Aβ contributes to neurodegeneration remains to be conclusively demonstrated, how the peptide is formed has been better characterized.

Aβ is formed by the proteolytic processing of its precursor protein, APP. APP is a membrane-spanning protein encoded for on chromosome 21. The APP gene product appears to be involved with synaptic transmission, axonal transport, cell adhesion and support, and cholesterol metabolism (for review, see Selkoe, 1994). Knockout (elimination of the gene product) and knockdown (reduction in the relative amount of a gene product) studies of APP have provided extensive information about its function. APP seems to play a role in muscle development or function, and has also been implicated in the formation of long-term potentiation (LTP), a cellular process argued to be essential for learning (Douglas & Goddard, 1975; Kauer et al., 1988; Dawson et al.,

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1999; Seabrook et al., 1999; Senechal et al., 2008). Under normal conditions, the APP protein is degraded via a series of events.

The proteolytic processing of APP results in Aβ fragments of varying length, depending on where APP is cleaved by specific enzymes called secretases (Shoji et al., 1992; Sisodia & Price, 1995). If APP is cleaved by alpha (α)-secretase, a soluble form of APP (sAPP) is secreted which is readily absorbed and processed by lysosomal proteolytic events. Cleavage by α-secretase occurs in the extracellular domain of APP within the Aβ sequence, thus preventing the formation of longer, neurotoxic forms of the Aβ peptide (Lannfelt et al., 1995). The senile plaques seen in AD are primarily made of the 40 and 42 amino acid Aβ peptides, with studies showing that the 42 amino acid Aβ is more neurotoxic, i.e. more damaging to neurons, than the shorter variants (Roher et al., 1996). If APP is cleaved by beta (β) and gamma (γ)-secretases, this leads to formation of an Aβ peptide 39-43 amino acids long, which is similar to the lengths of Aβ predominantly found in senile plaques in AD (Golde et al., 1992; Citron et al., 1996).

Initially, β-secretase (i.e. BACE) cleaves APP at its amino-terminus in the extracellular domain which is followed by cleavage by γ-secretase within the transmembrane domain (Vassar et al., 1999). BACE-1 inhibitors have been demonstrated to inhibit β-cleavage of APP and effectively lower Aβ levels *in vitro* and *in vivo* (Hussain et al., 2007). Inhibitors of γ-secretase have also been shown to reduce Aβ levels in the brain when administered to mice that overexpress a human mutant version of APP (Dovey et al., 2001). Inhibition of either of these two secretases theoretically leads to a reduction in the amount of Aβ because both β- and γ-secretase are necessary in order to cleave APP in a fashion that yields the 39-43 amino acid peptides known to aggregate

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and form plaques. Considerable pharmacological research is being directed at developing β- and γ-secretase inhibitors as therapeutic targets in an effort to reduce plaque load and perhaps even halt the progression of the disease. A great deal of research has also been conducted examining the genetic underpinnings of AD with a particular focus on genes tied to the proteolytic processing of Aβ.

Genetic linkage studies have tied familial forms of AD to the gene for APP on chromosome 21 (Goate et al., 1991; Citron et al., 1992). Over twenty mutations in the gene have been identified to date that are thought to be responsible for the familial early-onset form of the disease (Chai, 2007). Individuals with any of these mutations have a slightly increased chance, compared to the population as a whole, of developing early-onset AD because they have a greater amount of APP and thus produce more Aβ than normal individuals (Citron et al., 1992; Suzuki et al., 1994). Interestingly, almost all APP mutations are located within or adjacent to the Aβ peptide region of the precursor protein, and thus may affect the proteolytic processing of APP (Schellenberg, 1995). Related to the processing of APP are the presenilin (PS) genes which have also been implicated in autosomal dominant familial AD.

Presenilins have been linked to early-onset AD, specifically PS-1 on chromosome 14 and PS-2 on chromosome 1 (Levy-Lahad et al., 1995; Sherrington et al., 1995). Similar to the aforementioned mutations in APP, PS mutations lead to increased Aβ production, especially production of $A\beta_{42}$, a species of the peptide known to be most toxic to neurons and overabundant in AD (Iwatsubo et al., 1994; Duff et al., 1996; Citron et al., 1997). Investigations into PS mutations and Aβ deposition have shown that mutations in the PS gene increase the ratio of $A\beta_{42}$ versus $A\beta_{40}$ as compared to non-PS

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mutant cases of AD (Borchelt et al., 1996). This shift in the production of $A\beta_{42}$ can have significant consequences, as many studies have suggested that $A\beta_{42}$ aggregates more readily than $A\beta_{40}$ and is deposited early in the formation of plaques (Jarrett et al., 1993; Iwatsubo et al., 1994). Furthermore, PS mutations seem to alter APP or Aβ processing by increasing the amount of cleavage by γ-secretase, and thus increasing the amount of insoluble Aβ released (DeStrooper et al., 1998; Wolfe et al., 1999). Knockout studies with animals that do not express the PS-1 and PS-2 genes show an abolishment of γ-secretase mediated cleavage of APP (Steiner et al., 1999; Yu et al., 2001).

Based on these findings and others, Wolfe et al. (1999) proposed the hypothesis that PS itself is a γ-secretase, an intramembranous protease that is responsible for γ-cleavage of APP. Despite further evidence supporting this hypothesis, it appears that PS and γ-secretase are not the same protein even though they are highly related (Takasugi et al., 2003). Also, although PS-knockout mice seem to exhibit a complete abolishment of γ-secretase activity, these animals still produce $A\beta_{42}$ peptide fragments, suggesting there are additional enzymes with activity similar to γ-secretase (Wilson et al., 2002). While the discovery that these three genes (APP, PS-1, and PS-2) are linked to familial AD advanced the investigation of the disorder, it is important to recognize that they collectively only account for about 10% of all familial early-onset cases (Cruts et al., 1998). Given that more than 95% of AD cases occur after the age of 60, it is clear that these genetic mutations contribute only minimally to the risk of developing the more common variant of the disease (Holmes, 2002). With that said, much more is known about the genetics of familial early-onset AD than about sporadic, late-onset AD.

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One gene implicated in non-familial forms of AD is the gene coding for Apolipoprotein E (ApoE). ApoE is a protein critical in regulating brain Aβ peptide levels and trafficking lipids throughout the brain (for review, see Holtzman, 2001). ApoE is responsible for clearing Aβ peptides from the brain across the blood-brain barrier into the peripheral circulation (LaDu et al., 1994; LaDu et al., 1995; Morikawa et al., 2005). Aβ peptides are normally generated at very high levels in the brain and are cleared at an equivalent rate (Bateman et al., 2006). Thus, even small reductions in the clearance of Aβ could result in elevated levels of Aβ peptides and eventual plaque formation. The lipidation status of ApoE appears to be important with regard to how well it can bind to Aβ and clear the peptide from the brain (Tokuda et al., 2000). If ApoE is in a lipidated form, it is more effective at clearing Aβ than if it is non-lipidated.

Another way that Aβ is cleared from the brain is through a proteolytic mechanism involving either neprilysin (NEP) (Iwata et al., 2000) or insulin-degrading enzyme (IDE) (Kurochkin & Goto, 1994). Inhibition of either of these proteinases leads to a substantial elevation of Aβ levels in the brain and increased plaque deposition (Dolev and Michaelson, 2004). Recent research suggests that ApoE facilitates these enzymes, allowing them to degrade Aβ (Jiang et al., 2008). The ability of ApoE to clear Aβ is also dependent upon the isoform or allele of ApoE (Corder et al., 1993).

ApoE has three alleles: ApoE-ε2, ApoE-ε3, and ApoE-ε4, with one or two copies of the ε4 allele leading to an increased risk of developing AD (Corder et al., 1993). Studies with transgenic mice overexpressing APP have demonstrated ApoE isoform-specific effects on the ability of each allele to clear Aβ from the brain. The most effective allele at eliminating Aβ is the ε2 allele, followed by the ε3 allele, with the least effective

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being the ε4 allele (Holtzman, 2004). Thus, individuals with one or two ε4 alleles (two alleles being the least effective possible form) of ApoE have a less effective mechanism for clearing Aβ from the brain, resulting in increased levels of the peptide (Saunders et al., 1993). In fact, a genetic study done by Corder et al. (1993) showed that over 90% of subjects examined who had two copies of the ε4 allele (4/4) had AD. Almost 50% of subjects with one copy of the ε3 and one copy of the ε4 (3/4) were affected with AD. Only about 20% of subjects with no copies of the ε4 allele (2/2, 3/3, or 2/3) had the disease. Corder et al. (1993) also looked at the average age of onset in individuals with one or two copies of the ε4 allele. The authors found that expression of it leads to a significantly earlier age of onset, with two copies of the ε4 allele leading to an earlier onset than just one copy. While the discovery of the ApoE gene and its relation to Aβ is critical to understanding AD pathogenesis, it is not a determinant of the disease and it must be regarded carefully. A consequence of the Aβ pathology that arises due to these mutations may be an initiation of the inflammatory response, a common neurological occurrence in individuals with AD.

Glial cells in the brain, specifically microglia and astrocytes, can serve as mediators of the inflammatory response when necessary, defending the CNS from pathogens and aiding in the recovery from damage and stress (reviewed in Skaper, 2007). AD brains exhibit extensive localized activation of both microglia and astrocytes in response to neuronal and synaptic damage and Aβ accumulation (reviewed in Akiyama et al., 2000). It is likely this inflammation related to AD pathology may be both beneficial as a mechanism to promote neuronal survival and detrimental to AD brain function and the degeneration process (Wyss-Coray & Mucke, 2002). Recent studies have shown that

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aggregated Aβ is itself capable of activating the inflammatory response by activating microglia and enhancing the synthesis and release of proinflammatory cytokines (Tan et al., 1999; Combs et al., 2001). These proinflammatory cytokines may also accelerate tau pathology and NFT formation, perhaps linking Aβ-induced inflammation and neurofibrillary pathology (Sheng et al., 2000; Quintanilla et al., 2004; Guo et al., 2006). This finding would suggest that inflammation is actually advancing AD pathology and accelerating the neuronal loss. Although microglial activation in AD may be caused by Aβ pathology, microglial activity correlates more closely with NFT pathology (Hayes et al., 2002). This relationship suggests that although Aβ may trigger the initial activation of microglia, the resulting inflammatory response may be more directly related to tau pathology (Blurton-Jones & LaFerla, 2006). Another study done by Guo et al. (2006) suggests that soluble Aβ and tau may directly interact to promote each other's aggregation. In fact, it has been demonstrated that *in vitro* injection of Aβ activates GSK-3β, one of the enzymes thought to be responsible for phosphorylating tau (Lovestone et al., 1996). Thus, it is clear there is some relation between Aβ and tau pathology, but more studies are needed to clarify the connection.

Much of the above research eventually led to the amyloid cascade hypothesis which suggests that abnormal Aβ production and accumulation triggers the neurodegeneration seen in AD. Hardy & Higgins (1992) proposed that deposition of Aβ protein is the causative factor in AD pathology and that the NFT, neuronal loss, and dementia that follow are a result of this deposition. They hypothesized that neurotoxic Aβ peptides disrupt calcium homeostasis extracellularly and disrupt calcium concentrations intracellularly. This intra-neuronal increase in calcium concentration could potentially be

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what causes tau proteins within the cell to be hyperphosphorylated and form PHF, which are the primary component of NFT. Thus, for this interpretation of the amyloid cascade hypothesis to be correct, elevations in Aβ levels should cause hyperphosphorylation of tau and neurofibrillary pathology to develop. This link between Aβ and tau has been verified to some extent in animal models and in cell culture, wherein Aβ causes an increase in tau phosphorylation (Gotz et al., 2001; Lewis et al., 2001; Zheng et al., 2002). Transgenic mice harboring mutations in both human APP and human Tau, as well as a mutant PS-1 allele, dubbed 3xTg-AD mice, develop both Aβ deposits and NFT-like pathology (Oddo et al., 2003a). Studies using these mice have demonstrated that Aβ accumulation precedes the development of tau pathology by several months, further suggesting Aβ may promote tau phosphorylation and aggregation (Oddo et al., 2003b).

Despite the amount of research supporting the amyloid cascade hypothesis, there are several gaps between the hypothesis and data collected. First, the hypothesis is inconsistent with the presence of senile plaques in normal aged brains similar to those seen in AD (Crystal et al., 1988; Katzman et al., 1988; Price et al., 1991). If Aβ deposits are the catalyst for AD neurodegeneration, then we would not see individuals with no cognitive impairment and diffuse plaque load. Secondly, in transgenic animals that overexpress either APP or one of the PS mutations, there is no significant NFT formation or neurodegeneration despite considerable Aβ plaque load (Hsiao et al., 1996; Takeuchi et al., 2000). Because no AD-like pathology develops in these mice except for the plaques, it is difficult to argue that Aβ is directly causing the significant neuronal loss seen in the disease. Another limitation of the amyloid cascade hypothesis is that neurodegeneration and dementia occur in the absence of Aβ plaques in several diseases

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related to the tau protein (Hutton et al., 1998; Spillantini et al., 1998). Regardless of whether or not Aβ peptides are the causative agent in AD, it is likely that they contribute to the cognitive symptoms seen in the disease, as well as have some effect on the pathogenesis in AD. Another pathogenic contributor to the neurodegeneration observed in AD is the tau protein which is hyperphosphorylated resulting in NFTs and neuronal loss.

Tau Hypothesis

Tau is an intracellular protein found abundantly in the central and peripheral nervous systems and is critical for microtubule stability and assembly, as well as microtubule flexibility (Goedert et al., 1989; Goedert et al., 1992). Microtubules are located throughout the neuron and in the axon and are essential for neurotransmission, axonal transport and axonal support (Paulson & McClure, 1974; Weingarten et al., 1975; Kraemer et al., 2003). Tau is a phosphoprotein which implies that it requires phosphorylation, or the addition of a phosphate group, in order for it to become activated (Butler & Shelanski, 1986). Tau is partially phosphorylated in the normal brain and this phosphorylation may regulate microtubule stability and assembly. This microtubule regulation appears to happen by reducing tau's binding to tubulin, a protein that makes up microtubules, and reducing the promotion of microtubule assembly (Hasegawa, 2004). Thus, the phosphorylation of tau plays a pivotal role in regulating microtubule production by reducing the amount of microtubule assembly and decreasing the ability of tau to bind to tubulin.

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In AD, the tau protein is hyperphosphorylated, which leads to the destruction of microtubule assemblies via the aforementioned mechanisms (Grundke-Iqbal et al., 1986). The degradation of microtubules may cause impaired axonal transport and possibly cell death (Kosik et al., 1986). Tau hyperphosphorylation renders tau unable to bind to microtubules, an event proposed to be responsible for self-assembly into the paired helical filaments (PHFs) (Goedert et al., 1988; Bramblett et al., 1993; Yoshida & Ihara, 1993). These PHFs, which are primarily made up of hyperphosphorylated tau, correlate strongly with neuronal death in AD (Gomez-Isla et al., 1997). Also, whereas Aβ pathology is relatively specific to AD, NFT formation occurs in other diseases/disorders related to tau, collectively referred to as tauopathies.

In 1998, several mutations in the tau gene were discovered in families with frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), a related but distinct neurodegenerative disease, indicating genetic evidence that tau abnormalities may be sufficient to lead to neurodegeneration (Hong et al., 1998; Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998). This discovery led to the production of many different transgenic lines of mice with tau mutations. One specific line, the P301L mice, shows significant age-dependent NFT formation, memory impairment, and neuron loss (Lewis et al., 2000; Ramsden et al., 2005). Although there is significant neurodegeneration in these animals, they do not develop any Aβ pathology, implying that tau mutation or tangle formation is not sufficient on its own to cause senile plaques in an animal model of AD. These findings provide strong evidence that tau may induce neuronal loss in the absence of Aβ, but also suggest that Aβ plaque formation may lie upstream of tau, at least for AD.

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Consistent with the findings that Aβ plaque burden does not correlate well with the severity of dementia, but NFT formation does, is the finding that NFTs can be differentiated into neuropathological stages in AD. Braak & Braak (1991) reported detailed pathological studies about the distribution of plaques and tangles in autopsy brains of demented and non-demented individuals. They showed that Aβ deposition was of little significance in relation to neuropathological staging, whereas NFTs exhibited a neuroanatomical distribution pattern permitting the differentiation of six stages of disease progression in AD. Tangles are first observed in the entorhinal cortex where neuronal loss occurs the earliest, and are closely related to the initial memory impairment in AD, whereas Aβ deposits are not found in the hippocampal formation until the late stages of the disease (Hasegawa, 2004). Another post-mortem study showed that both NFTs and neuronal loss increased in parallel with the duration of AD, although the amount of neuronal loss was five or six times larger than the amount of tangle accumulation (Gomez-Isla et al., 1997). In contrast, the authors also found that the amount of plaques and Aβ accumulation were not related to neuronal loss, the number of NFTs, or the duration of the disease.

Despite the recent attention the tau hypothesis has received, it has limitations similar to the Aβ hypothesis. Foremost is the finding that NFTs are extremely common and perhaps universal in the nucleus basalis of Meynert, a basal forebrain region rich in cholinergic fibers, in non-demented aged individuals (Beach et al., 1998). Secondly, because animal models of tauopathies fail to develop Aβ pathology or the global neuronal loss seen in AD, it is difficult to claim that tau aggregation and NFTs are causing the disease. Another possible explanation for the significant neuronal loss and degeneration

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seen in AD is related to the cholinergic hypothesis and the loss of cholinergic neurons in areas important for learning and memory.

Cholinergic Hypothesis

The cholinergic deficit in AD is the earliest and most frequently reproduced finding, specifically the profound reduction in choline acetyltransferase (ChAT) activity (Davies & Maloney, 1976; Bowen et al., 1982). ChAT is the enzyme responsible for synthesizing acetylcholine (ACh), and its decreased activity leads to reduced amounts of available ACh in the brain, specifically in the hippocampus and neocortex (Perry et al., 1977; Davies, 1979). The finding that cholinergic cell loss is associated with the presence of plaques in non-demented aged individuals suggests that the loss of cholinergic neurons precedes the clinical diagnosis of AD (Beach et al., 1997). Furthermore, studies indicate that the severity of the dementia in AD correlates well with the extent of cholinergic loss and the reduction in ChAT activity in the cortex (Perry et al., 1981). Cholinergic loss occurs first and foremost in the basal forebrain where CBF neurons deteriorate very early in the disease progression (Whitehouse et al., 1982; Bowen et al., 1982; Beach et al., 2000). ACh has been implicated in different cognitive functions such as learning and memory (Whitehouse, 1967; Cox & Tye, 1973; Valentino & Dingledine, 1981; Spencer & Lal, 1983; Spencer et al., 1985; Woolf, 1996). Once ACh is released, it can bind to either of two receptor subtypes: nicotinic acetylcholine receptors or muscarinic acetylcholine receptors (Role & Berg, 1996; reviewed in Wevers & Schroder, 1999 and Ishii & Kurachi, 2003).

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Nicotinic receptors are ionotropic receptors (receptors that can open ion channels when ACh binds to them) while muscarinic receptors are metabotropic receptors (receptors that activate a G-protein and trigger intracellular events when ACh binds, including the opening of multiple ion channels). There appears to be a selective loss of ACh receptors in the cortex and hippocampus in AD, and it seems to be more pronounced for nicotinic receptors (Flynn & Mash, 1986; Perry et al., 1995; Wevers et al., 2000).

Nicotinic receptor activation using nicotine produces a significant increase in the amount of phosphorylated tau both *in vitro* and in 3xTg-AD mice (Hellstrom-Lindahl et al., 2000; Oddo et al., 2005). The exact mechanism of how this increase in phosphorylation occurs remains unclear, but it may be a result of increased calcium levels due to overactivation of the nicotinic receptors. Nicotonic receptors are one of only a few ionotropic receptors in the brain that allow an influx of calcium ions when ACh binds (McGehee et al., 1995; Role & Berg, 1996). This increase in intracellular calcium may activate different calcium-dependent kinases, such as GSK3β or p38MAP kinase, which may be responsible for phosphorylating tau (Oddo et al., 2005). Nicotinic receptor activation also appears to have an effect on Aβ deposits in the brain. *In vitro* studies have shown that nicotine seems to inhibit Aβ fibril formation and also disrupts already formed fibrils (Salomon et al., 1996; Zeng et al., 2001; Ono et al., 2002). This would suggest that in the absence of ACh, the formation of fibrils is allowed to progress at a more rapid rate. Studies using transgenic mice, specifically mice that overexpress human APP called Tg2576, showed a dramatic decrease both in Aβ plaque burden and the levels of insoluble Aβ40 and Aβ42 after chronic administration of nicotine for a period of five and a half months (Nordberg et al., 2002). It is difficult to determine what effect the loss of

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nicotinic receptors has on tau phosphorylation and Aβ deposits in AD, and more studies are necessary to elucidate the relationships.

Muscarinic receptors, specifically the M1 receptor subtype, are highly expressed in the cerebral cortex and hippocampus, and seem to be particularly relevant to memory function in AD (Anagnostaras et al., 2003). The M1 receptor has been shown to decrease tau phosphorylation suggesting that decreased cholinergic activity may lead to destabilization of the microtubule network and eventual tangle formation. *In vitro* studies using cholinergic M1 agonists showed that the muscarinic-activated decrease of tau phosphorylation was both time and dose dependent (Sadot et al., 1996). *In vivo* studies using 3xTg-AD mice also showed a reduction in tau phosphorylation after administration of a muscarinic agonist and conversely showed increased tau phosphorylation after treatment with an M1 antagonist (Caccamo et al., 2006). Previous *in vitro* findings found that M1 receptor agonists decrease tau phosphorylation by the reduction of GSK3β activity (Forlenza et al., 2000), which appears to be the mechanism by which tau phosphorylation was decreased in the 3xTg-AD mice. Muscarinic receptor activation also appears to reduce Aβ production and increase the secretion of soluble APP (Buxbaum et al., 1992). In the 3xTg-AD mice, an M1 agonist reduced Aβ deposition in the hippocampus and cortex, and ameliorated cognitive deficits in a spatial memory task (Caccamo et al., 2006). This finding that muscarinic activation regulates APP processing (Nitsch et al., 1992) formed the basis for the hypothesis that AChE inhibitors may slow disease progression by reducing Aβ production (Inestrosa et al., 1996).

AChE inhibitors were developed as a result of the cholinergic hypothesis in order to increase cholinergic tone in individuals with AD (Davis et al., 1978; Bartus, 1979;

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Bartus et al., 1982). AChE is primarily responsible for the breakdown of ACh into choline and acetic acid. AChE has also been implicated in Aβ plaque formation and appears to have the ability to accelerate Aβ formation and deposition in AD (Inestrosa et al., 1996). Therefore, by inhibiting the enzymatic activity of AChE, it may be possible to reduce Aβ plaque formation and ameliorate AD symptomology. AChE inhibitors increase the amount of ACh available in the synapse as well as enhance and prolong its action on ACh receptors (Harvey & Rowan, 1990). AChE inhibitors have been approved for use in mild to moderate AD and have been shown to improve cognitive deficits (Rogers et al., 1998; Rosler et al., 1999; Tariot et al., 2000). One of the most widely prescribed and used AChE inhibitors is donepezil.

Donepezil hydrochloride is a piperidine-based, non-competitive, reversible inhibitor of AChE with high central nervous system specificity and a long duration of action (Yamanishi et al., 1991). It has been demonstrated to be a well-tolerated drug that improves cognitive performance and global function in mild to moderate AD patients (Rogers et al., 1996; Rogers et al., 1998). Donepezil significantly increases extracellular ACh concentrations both dose- and time-dependently (Kosasa et al., 1999). Furthermore, donepezil was able to significantly improve a deficit in spatial learning and memory induced in rats with lesions of the medial septum, a pathway by which cholinergic neurons project to the hippocampus, at a relatively low dose of 0.5 mg/kg. The rescue of the spatial deficit was almost to the level of the control animals in a Morris water maze (MWM) spatial learning task (Ogura et al., 2000). Scopolamine, a cholinergic antagonist, has been shown to produce learning impairments in rodents (Ogren et al., 1998; Elvander et al., 2004). Donepezil, once again at a low dose of 0.5 mg/kg, was able to significantly

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decrease the scopolamine-induced deficit in rats in an 8-arm radial arm maze, a spatial learning and memory task (Ogura et al., 2000).

Many animal models of AD show Aβ pathology and a few even show tau pathology, but thus far none have been able to replicate the global neuronal loss exhibited in AD. Nevertheless, administration of donepezil to different AD models provides valuable insight into the cellular and molecular effects of the drug, as well as examines the effect it has on behavioral deficits. One such animal model involves lesions of the entorhinal cortex, a part of the brain which provides inputs to the hippocampus, using ibotenic acid, a compound that produces non-specific excitotoxic lesions. Although the lesion does not model the specific cholinergic loss which is a hallmark of AD, it is a valuable tool to study hippocampal learning and memory deficits. One study administered donepezil following an ibotenic acid lesion and found that donepezil was able to partially rescue the lesion-induced deficit in the MWM (Spowart-Manning & van der Staay, 2005).

Further animal models are the Tg2576 mice and APP23 mice that overexpress human APP containing the Swedish double mutation and display age-related cognitive deficits and plaque load (Hsiao et al., 1996; Sturchler-Pierrat et al., 1997; Kelly et al., 2003). Briefly, the gene coding for APP is mutated to overexpress the protein and the mice eventually develop Aβ plaques around 6 months of age (Sturchler-Pierrat et al., 1997). A study with the Tg2576 mice showed that donepezil administration dose-dependently (0.1, 0.3, or 1.0 mg/kg) reduced the cognitive deficit in a spatial memory task (Dong et al., 2005). Another study investigated the effect of donepezil administration (0.3 mg/kg) in the APP23 transgenic mice and found that it was able to rescue some of

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the deficits in the MWM (Van Dam et al., 2005). In a follow-up study using the APP23 mice, Van Dam et al. (2008) found that donepezil may have disease-modifying effects. After chronic donepezil treatment (0.27 mg/kg per day for 8 weeks), animals experienced a 3 week wash-out period where no drug was given in order to determine if treatment was able to improve spatial memory in the MWM after the drug was no longer active. Donepezil significantly improved performance of the APP23 mice both in acquisition and retention of spatial memory in the MWM. This possible disease-modifying effect of donepezil may be due to the relation between AChE and Aβ formation that we previously reviewed. Because AChE has been implicated in amyloid plaque formation (Inestrosa et al., 1996), the inhibition of AChE may lead to reduced Aβ pathology. In fact, donepezil *in vitro* reduced AChE-induced Aβ aggregation by 22% (Bartolini et al., 2003). Further evidence from *in vitro* studies found donepezil, but not other AChE inhibitors, had neuroprotective effects on cell culture models of neuronal injury (Akasofu et al., 2008).

Another possible explanation for donepezil's possible disease-modifying ability is that in addition to its action on AChE, it antagonizes N-methyl-D-aspartate (NMDA) receptors in the brain (Wang et al., 1999; Moriguchi et al., 2005). NMDA receptors are activated by glutamate, an excitatory neurotransmitter, and when NMDA is open it allows calcium ions into the cell. An overabundance of calcium ions can lead to excitotoxicity and neuronal death which is what may be occurring in AD (Lucas & Newhouse, 1957; Mattson et al., 1992). Thus, because donepezil is able to reduce the activity of the NMDA receptor, it also may reduce excitotoxicity. This excitotoxic inhibition may be mediated through nicotinic ACh receptors followed by specific kinases which reduce NMDA receptor activity (Takada-Takatori et al., 2006). Interestingly,

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donepezil has also been found to improve cognitive performance in healthy young rats (Cutuli et al., 2008), which suggests donepezil may act as a cognitive enhancing drug in normal animals as well as in animals with cognitive deficits.

Donepezil is clearly able to ameliorate deficits related to AD pathology in early and middle stages of the disease in AD patients and in animal models (Rogers et al., 2000). It increases cholinergic tone and activity, likely compensating for the profound deterioration of CBF neurons in AD. These findings also indicate why in the latter stages of AD donepezil has been found to be less effective, as the overall loss of cholinergic neurons may progress to a level beyond the capacity of an AChE inhibitor to improve. Although it is not yet evident why these CBF neurons begin dying in AD, one possible explanation is related to calcium levels and excitotoxicity.

Excitotoxicity Approach

Glutamate is an excitatory neurotransmitter pervasively expressed throughout the central nervous system (Harvey & McIlwain, 1968). It can act on either ionotropic receptors such as AMPA, kainate or NMDA receptors, or metabotropic receptors which are coupled to G-proteins (Ishida & Neyton, 1985; O'Brien & Fischbach, 1986; Cull-Candy & Usowicz, 1987; Jahr & Stevens, 1987). Binding of glutamate to NMDA receptors leads to an influx of calcium and excitation (MacDermott et al., 1986). Because glutamate is a potent excitatory transmitter, excessive amounts or prolonged release can elicit excessive excitation in neurons that may lead to cell death (Lucas & Newhouse, 1957; Olney & Ho, 1970). Chronic exposure to moderately elevated glutamate levels or hyperactivity of glutamate receptors, which is what may be occurring in many

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neurodegenerative diseases as well as cerebral ischemia, can lead to a steady influx of calcium which has been shown to be toxic for the cell (reviewed in Mattson & Chan, 2003). This calcium-induced cell death, also called excitotoxicity, has been investigated in AD and may play a role in the progressive degeneration of neurons.

Calcium levels in AD appear to be elevated, potentially resulting in excitotoxicity and cell death (Peterson et al., 1985; Peterson & Goldman, 1986). Calcium levels are increased in neurons that contain NFTs as compared with tangle-free neurons (Murray et al., 1992). The increased levels of calcium may precede tangle formation based on the finding that levels of calcium/calmodulin-dependent protein kinase II (CamKII), the most abundant kinase in the brain and dependent on calcium for activation, are increased in hippocampal neurons which are vulnerable to degeneration (McKee et al., 1990). Calcium levels may also be affected by Aβ peptides. *In vitro* studies show that application of Aβ peptide increases the vulnerability of cortical neurons to glutamate toxicity (Mattson et al., 1992). *In vivo*, the toxicity caused by Aβ injection into the hippocampus or nucleus basalis of Meynert is reduced by NMDA antagonists, showing that the toxicity is in part related to glutamatergic activity (Harkany et al., 1999; Miguel-Hidalgo et al., 2002). These studies suggest that glutamate receptor activation and exposure to Aβ peptides together are more injurious than either insult alone (Canzoniero & Snider, 2005). In addition to increasing vulnerability to calcium influx through glutamate receptors, specifically NMDA, Aβ can also increase resting calcium levels both intra- and extracellularly (Mattson et al., 1992). In an attempt to address the excitotoxic damage caused by increased calcium levels and overactivity of glutamate receptors, NMDA-receptor antagonists have been developed.

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Memantine is an uncompetitive NMDA antagonist that has been approved for use in moderate to severe cases of AD dementia (Ditzler, 1991; Winblad & Poritis, 1999; Tariot et al., 2004). What makes memantine an uncompetitive antagonist is that it becomes more effective at blocking NMDA receptors the more the receptors become activated. In other words, under normal physiological conditions, the NMDA receptor will not be antagonized by memantine because the drug will remain inactive. When the NMDA receptor is overactivated by glutamate, memantine becomes extremely effective at returning NMDA activity to a normal state. At a fixed concentration of memantine, its ability to block the NMDA receptor increased as the concentration of NMDA increased (Chen et al., 1992). For this reason memantine is usually only administered in later stages of AD when calcium concentrations may be sufficiently elevated and the drug is able to exert its unique uncompetitive NMDA blockade. Another way of possibly reducing the excitotoxicity seen in AD is via the endogenous peptide galanin, which has been shown to exhibit neuroprotective effects.

Galanin Literature

Galanin is a 29 amino acid (30 in humans) neuropeptide, first isolated from porcine intestine by Tatemoto et al. (1983). It is widely expressed throughout the mammalian nervous system with galaninergic neurons in areas such as the cerebral cortex, nucleus basalis of Meynert, hippocampus, amygdala, and locus coeruleus (Kordower et al., 1992; Perez et al., 2001). Galanin inhibits several classical neurotransmitters including glutamate, serotonin, norepinephrine, and ACh (Dutar et al., 1989; Pieribone et al., 1995; Robinson et al., 1996; Kinney et al., 1998; Ogren et al.,

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1998; Xu et al., 1998). In the basal forebrain of rodents, galanin co-localizes with cholinergic neurons within the medial septum/diagonal band complex (Melander et al., 1985; Melander et al., 1986; Miller et al., 1998). In the human basal forebrain, there exists a small population of non-cholinergic galaninergic interneurons and a dense galaninergic fiber plexus that innervates CBF neurons (Mufson et al., 1993; Bowser et al., 1997). In fact, it was found that galanin immunoreactivity is present in ~50-70% of CBF neurons (Melander et al., 1985). These findings led to the hypothesis that galanin may mediate the cholinergic system related to cognitive function (Mufson et al., 1998). *In vitro* studies have demonstrated that galanin reduces ACh release and ACh-elicited excitation and also inhibits LTP in hippocampal neurons (Fisone et al., 1987; Dutar et al., 1989; Palazzi et al., 1991; Sakurai et al., 1996). A different *in vitro* study found that galanin actually has the ability to excite cholinergic neurons, but further studies are needed to clarify this excitatory role for galanin (Jhamandas et al., 2002). *In vivo* work has shown that galanin administered into the hippocampus, lateral ventricles, or basal forebrain impairs cognitive performance on spatial learning and memory tasks in rats (McDonald et al., 1998; Wrenn & Crawley, 2001; Kinney et al., 2003). Furthermore, mice that overexpress galanin (GalOE) display cognitive deficits characteristic of AD (Steiner et al., 2001). Taken together, these data suggest that galanin may inhibit the basal forebrain and hippocampus to regulate cognitive processes.

Alternatively, galanin also appears to play a neurotrophic or neuroprotective role in the brain (Holmes et al., 2000; O'Meara et al., 2000; Mahoney et al., 2003; Elliot-Hunt et al., 2004; Elliot-Hunt et al., 2007). Following trauma or injury to the central nervous system, galanin expression is up-regulated in many brain regions (Cortes et al., 1990).

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Galanin also acts as a survival and growth-promoting factor to neurons in both the central and peripheral nervous systems (Holmes et al., 2000; O'Meara et al., 2000; Mahoney et al., 2003). Zini et al. (1993) found that galanin reduces glutamate release by 50-60% in rat hippocampal slices. Because glutamate is the principle excitatory neurotransmitter in the brain, and it can be responsible for calcium-induced excitotoxicity, the ability of galanin to reduce glutamate release classifies it as a trophic factor. Elliot-Hunt et al. (2004) also found that galanin promotes hippocampal neuron survival in a number of *in vitro* models of excitotoxic injury. Using hippocampal slices from transgenic animals either over-expressing galanin (GalOE) or galanin knockout (GalKO), galanin was shown to exert a protective effect after administration of glutamate or staurosporine, an agent which inhibits protein kinase activity and can lead to apoptosis, or programmed cell death. Galanin has also been shown to protect neurons from the toxic effects of Aβ *in vitro*, further exhibiting its neuroprotective capacity (Ding et al., 2006). Due to this ability to reduce excitotoxic damage and protect from neuronal injury, galanin agonists have significant implications as anti-epileptic agents. In order to further understand galanin and its possible therapeutic effects, we must first examine its receptors which would ultimately be the targets of any drugs.

Galanin receptors are G-protein-coupled metabotropic receptors, which activate second messenger pathways after the binding of a ligand (Hulting et al., 1993; Walli et al., 1994; Branchek et al., 1998). The first galanin receptor (GAL-R1) is a $G_{i/o}$ coupled receptor, which belongs to a family of receptors with mostly inhibitory actions. GAL-R1's are found throughout the human brain, particularly in the cortex, hippocampus, thalamus, and amygdala (Habert-Ortoli et al., 1994). Activation of GAL-R1 leads to

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inhibition of adenylyl cyclase and cyclic adenosine monophosphate (cAMP), both of which are signal transduction mechanisms crucial for learning and memory processes, especially consolidation of memory (Palazzi et al., 1991; Karelson & Langel, 1998; Wang et al., 1998; Iismaa & Shine, 1999; Kinney et al., 2003).

The second galanin receptor subtype (GAL-R2) can be of a G_q subtype or a $G_{i/o}$ subtype, and can be either excitatory or inhibitory (Fathi et al., 1998; Wittau et al., 2000). GAL-R2's are found in areas of the human brain similar to GAL-R1's including the cortex, hippocampus, amygdala, thalamus, and cerebellum (Borowsky et al., 1998; Fathi et al., 1998). A key difference between the two receptor subtypes which may explain their different mechanisms of action is that they are found in different parts of the hippocampus and exert different effects when activated. The ventral hippocampus contains significantly more GAL-R1's than the dorsal hippocampus which contains more GAL-R2's (Melander et al., 1985; Melander et al., 1986; Fisone et al., 1987; O'Donnell et al., 1999). Accordingly, when galanin is injected directly into the ventral hippocampus where there is higher GAL-R1 expression, learning and memory was impaired in the Morris water task. Conversely, when galanin is injected into the dorsal hippocampus, no learning deficits were detected (Ogren et al., 1999). GAL-R2 activation appears to increase the levels of inositol phosphates and intracellular calcium in cell cultures, leading to increased LTP and synaptic plasticity (Fathi et al., 1998; Kolakowski et al., 1998; Wang et al., 1998; Wittau et al., 2000). Thus, GAL-R2's appear to activate phospholipase C and protein kinase C, initiating a signal transduction pathway which promotes neurotransmission and facilitates learning and memory. Furthermore, activation

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of the GAL-R2 protects the hippocampus from neuronal damage, implicating this receptor subtype in the trophic role galanin plays (Elliot-Hunt et al., 2007).

The third galanin receptor subtype (GAL-R3) appears to be similar to GAL-R1 in functionality, coupling to the G_i protein, however the prevalence of GAL-R3 within the CNS is much less extensive and considerably more studies are needed on this receptor (Kolakowski et al., 1998; Smith et al., 1998). Thus, depending on which galanin receptor is activated, the effect may be inhibitory or excitatory; a distinction necessary to make in order to understand what is occurring in AD. In AD, the overexpression of galanin may be inhibitory, further exacerbating cognitive deficits, or it may be neuroprotective, attempting to save CBF neurons from any further damage.

AD patients exhibit characteristic memory loss and learning deficits, especially in later stages of the disease. When galanin is centrally administered to rodents, it causes learning and memory deficits, specifically deficits in the consolidation of memory (Kinney et al., 2003). Because of this finding and others like it, it can be said that it is possible that the overexpression of galanin seen in AD is contributing to the cognitive impairments exhibited in the disease. The mechanism by which galanin may contribute to the cognitive impairment has not been clarified, although many findings suggest it is through interactions with the cholinergic system (Fisone et al., 1987; Chan-Palay, 1988; Dutar et al., 1989; Mufson et al., 1993).

The loss of CBF neurons in AD is accompanied by an increase in the activity of the surviving cholinergic neurons (McDonald & Crawley, 1997). Because of galanin's role as an inhibitory neuromodulator, this increase in cholinergic activity may increase galaninergic activity, thus inhibiting the remaining cholinergic neurons (Fisone et al.,

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1987; Chan-Palay, 1988; Chan-Palay, 1990; Mufson et al., 1993; Bowser et al., 1997). The inhibition of surviving cholinergic neurons may then lead to the learning and memory problems characteristic of late-stage AD, because of the aforementioned importance of acetylcholine in learning and attentional processes. This theory is supported by findings that galanin expression progressively increases in the basal forebrain in AD (Mufson et al., 2000). Galaninergic fibers form a dense plexus surrounding surviving CBF neurons, reaching concentrations twice that of age-matched controls (Beal et al., 1990). Although there is evidence of increased galaninergic activity early in the disease progression (Perez et al., 2002), it appears that galanin hyperinnervation of CBF neurons only occurs in the late stage of the disease (Chan-Palay, 1988; Mufson et al., 1993; Bowser et al., 1997; Mufson et al., 2000; Counts et al., 2006). These findings are consistent with the notion that galanin overexpression is triggered by neuronal damage, suggesting galanin hyperinnervation of CBF neurons represents a neuronal survival mechanism (Gabriel et al., 1995; Hartonian et al., 2002; Shen et al., 2003).

In a recent study examining tissue from AD brains, it was found that CBF neurons that are hyperinnervated by galanin show a significant increase in choline acetyltransferase (ChAT) expression as compared to CBF neurons that have no GAL hyperinnervation (Counts et al., 2008). The increase in ChAT due to galaninergic hyperinnervation observed in this study suggests a neuroprotective role for galanin in AD. In a study using saporin to produce a cholinergic-specific lesion in the basal forebrain of rats, it was found that galanin immunoreactivity within and adjacent to the lesion was significantly increased as compared to control animals, an effect that persisted

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long after the treatment (Hartonian et al., 2002). Thus, damage to cholinergic neurons would appear to be sufficient to increase galanin expression long-term as a trophic response to the damage. However, in Down's syndrome which also produces extensive CBF degeneration, galanin does not hyperinnervate surviving cholinergic neurons (Mufson et al., 1993; Sendera et al., 2000). These findings suggest that CBF neuron loss alone is insufficient to trigger the galanin plasticity response seen in AD. Therefore, it may be that galanin is overexpressed in AD due to other factors such as the Aβ plaques or the NFT or the excitotoxicity. Whether galanin is protecting or inhibiting CBF neurons in AD is still unknown, but it is clear the neuropeptide needs to be studied further in order to fully understand its role in this neurodegenerative disease.

Hypothesis and Implications

Many theories have been proposed to explain the neurodegeneration which occurs in AD, but none so far have been able to account for the massive neuronal loss and progressive cognitive decline seen in the disease. Galanin may provide a critical link in the causal chain of AD, and thus the current study has potential implications for future Alzheimer's research. The effects of the co-administration of donepezil and galanin should provide valuable insight into AD pathology and further supplement the literature related to ACh and galanin.

In a series of experiments we attempted to determine if galanin exerts its effects on learning and memory via modulation of cholinergic tone in an intact nervous system, as well as to examine its effects in an animal model of AD when co-administered with a drug that is used to treat the disorder. In experiment 1, we investigated the effects of

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galanin when co-administered with donepezil in a spatial learning task, the Morris water maze, in an effort to clarify the mechanism by which galanin impairs learning and memory. If the galanin-induced deficit is altered by the addition of donepezil, it is likely galanin is impairing learning and memory in part via inhibition of cholinergic mechanisms. However, if donepezil has no effect on the galanin-induced deficit, then the deficit is likely unrelated to cholinergic alterations. Recent data from our lab indicate a drastic reduction in CREB (cAMP-response element binding protein) phosphorylation, a critical event for the induction of LTP and learning and memory (Comb et al., 1986; Dash et al., 1990; Bartsch et al., 1998), following galanin administration (Kinney et al., 2009). In addition, these changes have been linked to the learning impairments observed following exogenous galanin administration (Kinney et al., 2003). Therefore, we hypothesized that donepezil would be unable to rescue the galanin-induced deficit, and that galanin's inhibition of CREB phosphorylation may be principally responsible for the learning deficit.

There is a paucity of evidence on the role galanin may play following a cholinergic lesion with regard to both the survivability of cholinergic neurons and learning and memory. In experiment 2, we examined the effects of donepezil and galanin following the central administration of a cholinergic-specific neurotoxin, AF64A, designed to mimic an early or even preclinical stage of AD. While previous investigations have been carried out on either the effectiveness of AChEIs following a cholinergic lesion or the potential role of galanin in AD, there have been no studies examining both galanin and AChEIs in a model consistent with AD. Whether or not galanin functions in a neuroprotective capacity following a cholinergic lesion as well as

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how it affects learning following damage are key concepts that have yet to be addressed. In addition, we sought to determine what effect the co-administration of donepezil and galanin would have in a compromised cholinergic system. We hypothesized that donepezil would rescue any lesion-induced learning and memory deficits by increasing the amount of available ACh and restoring the functioning of the cholinergic system to a physiologically normative level. We further hypothesized that although galanin may not rescue the lesion-induced impairment, it may potentially reduce the degree of cell loss in these animals if it does act as a trophic factor *in vivo*. Finally, we expected that co-administration of donepezil and galanin would lead to a partial rescue of the learning and memory deficits caused by AF64A. However, we hypothesized that the inhibitory effects of galanin would not allow a complete reversal of the lesion-induced deficits.

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CHAPTER 3

MATERIALS AND METHODS

Subjects

Ninety adult male Sprague-Dawley rats (forty for Experiment 1 and fifty for Experiment 2) approximately three months of age and weighing between 250 and 350 g were used. Rats were maintained in a temperature and humidity (22 ± 1°C) controlled facility, with food and water available ad libitum, on a 12:12 light/dark cycle, lights on at 7:00 a.m. Animals were housed in pairs until the time of the surgery, after which they were individually housed. All procedures were approved by the University of Nevada, Las Vegas Institutional Animal Care and Use Committee and carried out in accordance with NIH guidelines for the appropriate care and use of animals.

Surgery

Surgeries were performed as described previously by Kinney et al. (2003). Briefly, all animals underwent stereotaxic surgery under aseptic conditions and ketamine (66 mg/kg; Henry Schein Inc, Sparks, NV) and dexmedetomidine (0.2 mg/kg; Henry Schein Inc, Sparks, NV) anesthesia. A guide cannula 1.4 cm in length, 24-gauge stainless steel hypodermic tubing (Plastics One, Roanoke, VA) was implanted into the right lateral ventricle at coordinates 0.5 mm posterior, 1.2 mm lateral to bregma, and 3.5 mm ventral to the surface of the skull (Paxinos & Watson, 1986). The cannula was secured to the skull using stainless steel screws and dental acrylic. A 31-gauge stylet was inserted to close the guide cannula. Rats were administered an analgesic (Buprenorphine, 0.05

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mg/kg; Henry Schein Inc, Sparks, NV) immediately following surgery as well for two days post-surgery to minimize post-operative pain.

Drug Treatments

Rat galanin 1-29 (Bachem Americas, Inc. Torrance, CA) was dissolved in 0.9% physiological saline vehicle at a concentration of 3 nmol/3 µl. Donepezil hydrochloride (Tecoland Corporation, Edison, NJ) was dissolved in 0.9% physiological saline to a concentration of 0.3 mg/kg. AF64A was dissolved in NaOh to a concentration of 0.34 mg/ml and pH was adjusted to between 7.0 and 7.4. The toxin was administered into the right lateral ventricle, 2 µl total, over the course of one minute with the injector being left in for an additional minute to ensure complete distribution of the solution. Subjects were randomly assigned to different treatment groups, each of which consisted of a daily pre-treatment administered intra-peritoneally (i.p. 1 ml/kg) and a daily treatment administered intracerebroventricularly (i.c.v. 3 µl infused). The pre-treatment was given 20 minutes prior to behavioral testing and the treatment was given 5 minutes before testing. Saline was administered as a control for i.p. pre-treatments and artificial cerebrospinal fluid (ACSF) was administered as a control for i.c.v. treatments.

For the first experiment, a control group received a saline pre-treatment and a treatment of ACSF. The remaining three groups received daily administrations of either a donepezil pre-treatment and a treatment of ACSF, a saline pre-treatment and a treatment of galanin, or a pre-treatment of donepezil and a treatment of galanin. In Experiment 2, four of the five groups received a one-time infusion of AF64A an average of four days before behavioral testing began. A control group received a sham-lesion, using ACSF

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instead of the toxin, and a pre-treatment of saline followed by treatment with ACSF. The remaining toxin-infused groups received daily administrations of either a pre-treatment of saline and a treatment of ACSF, pre-treatment of donepezil and a treatment of ACSF, pre-treatment of saline and a treatment of galanin, or pre-treatment of donepezil and a treatment of galanin. Table 1 outlines the drug administrations for each group in experiments 1 and 2.

Table 1

Experiment 1

Pre-treatment	Treatment
Saline	ACSF
Donepezil	ACSF
Saline	Galanin
Donepezil	Galanin

Experiment 2

Before Testing	Pre-treatment	Treatment
Sham lesion	Saline	ACSF
Lesion	Saline	ACSF
Lesion	Donepezil	ACSF
Lesion	Saline	Galanin
Lesion	Donepezil	Galanin

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Morris Water Task

The Morris water task was conducted in a circular tank, 1.8 m in diameter and 76 cm in height, made of white polyethylene 4.7 mm in thickness (San Diego Instruments, San Diego, CA). Tap water, 48 cm deep, was maintained at a temperature of 25°C and made opaque by the addition of white non-toxic paint (Fresco Tempera Paint, Rich Art Color Company, Northvale, NJ), and changed every other day. The escape platform, a square platform 10 cm in diameter made of clear plastic, was placed in the center of one of the four quadrants (target quadrant), 30 cm from the inside wall of the maze and 1.5 cm below the surface of the water. For visible platform training, a large black and white cover was attached to the top of the platform and protruded 2 cm above the water.

Trials were recorded and captured using a video tracking system (Smart, San Diego Instruments, San Diego, CA) recorded from a Sony Handycam camera connected to a Cobalt Instruments computer. Data collected for each trial consisted of a track of the animal which included the latency to locate the platform, speed of swimming, and thigmotaxis. On the probe trial the tracking system also recorded the amount of time subjects spent in each of the four quadrants of the maze, as well as the number of times the animal's path crossed over the previous platform location and its analogous location in each quadrant.

Behavioral Testing

Animals received AF64A toxin infusions an average of four days before testing began in order to alter the cholinergic system consistent with early stages of AD. Daily

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injections of donepezil or saline were administered 20 minutes prior to start of testing, while galanin or ACSF was centrally infused 5 minutes before testing began. Subjects were then taken individually from the colony room to a dedicated testing room containing the water maze, a computer desk, a table with the heating cage, and large geometric shapes positioned on each of the four walls, all serving as distal spatial cues. The rat was placed into the maze at one of three randomized locations, in the center of a quadrant that did not contain the escape platform (non-target quadrant). The rat was allowed to swim in the maze until it reached the hidden platform and placed its forepaws on the platform. If after 60 seconds the animal did not locate the hidden platform, it was guided to the platform by the experimenter. The rat was given 20 seconds on the platform to orient to distal spatial cues and was then placed under a heat lamp for a total of 30 seconds between trials. Three additional trials were conducted in an identical fashion, for a total of four training trials per day. Following the fourth trial, the animal was dried and then returned to its home cage. The training trials for the hidden platform were conducted until control subjects reached a latency criterion of less than 20 seconds for experiment 1 and less than 13 seconds for experiment 2. A more stringent criterion was set for experiment 2 in order to ensure lesion animals had sufficient opportunity to learn the task. A probe trial was conducted five hours following achievement of this criterion. For probe trials, the rat was placed in the maze in the same fashion as during training, but the escape platform was absent. The single probe trial was 60 seconds in duration, after which the rat was dried and returned to its home cage.

The day after completion of the probe trial, a visible platform training protocol was used. A visible platform that extends above the surface of the water (intra-maze cue)

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was placed into the maze instead of the hidden platform. Five trials were conducted for each animal in the same fashion as during the hidden platform training, with the exception that the platform location was changed on each trial. Visible platform training was conducted in order to detect any deficits in visual ability and motor function. Thirty minutes following completion of the last visible training trial, animals were humanely euthanized.

Tissue Collection

Animals were humanely euthanized via carbon dioxide asphyxiation and half were transcardially perfused while the other half were immediately decapitated for rapid removal of the brain. Transcardiac perfusions involved perfusing saline through the vascular system via the left ventricle followed by perfusion of 8% paraformaldehyde to fix the tissue. Brains were then removed and placed in 8% paraformaldehyde at 4° C for 48 hours followed by 24 hours in a 30% sucrose solution in PBS (phosphate-buffered saline). Finally, brains were placed in 5% sucrose in PBS until immunohistochemistry experiments. For rats that did not receive transcardiac perfusions, brains were quickly removed and cortex, hippocampi, and cerebellum were dissected out and flash frozen in dry ice. The dissected tissue was stored at -80° C until western blotting experiments.

SDS-PAGE (Western Blots)

A total of twenty-four animals were randomly selected for the SDS-PAGE experiments which were only conducted with subjects from experiment 2. Tissue was homogenized in a non-denaturing lysis buffer consisting of 1X RIPA buffer (Cell

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Signaling; 20 mM Tris-HCL pH 7.5, 150 mM NaCL, 1 mM Na_2 EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and 1 µg/ml leupeptin), 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 µg/ml aprotinin and 0.1% sodium dodecyl sulfate (SDS). Lysates were centrifuged at 15,000 x g for 15 minutes at 4°C, the supernatant was collected, and a protein assay to determine concentration was performed using the biciconinic acid method (BCA, Pierce, Rockford, IL). Samples (20 µg) were separated on 10% SDS-PAGE gels according to the method of Laemmli (1970). Proteins were then electro-transferred to nitropure 45 micron nitrocellulose membranes which were blocked in 5% milk in Tris-buffered saline 0.05% Tween (TBST) and sodium azide overnight.

Individual membranes were incubated with rabbit anti-VAChT (vesicular ACh transporter) antibody (1:750; Sigma-Aldrich, St. Louis, MO) or rabbit anti-β-actin antibody as control (1:1000; Sigma-Aldrich, St. Louis, MO) in TBST-5% milk plus sodium azide for 2 hours at room temperature. Detection of specific binding was performed by incubation with HRP-conjugated secondary antibodies (1:5000, Vector) for 1 hour at room temperature. Specific signals were detected with Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and images were then scanned for densitometry, using BioSpectrum Imaging System (UVP, Upland, CA) and average intensity was obtained for each sample using ImageJ software (National Institutes of Health).

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Immunohistochemistry

Twenty-three animals, all from experiment 2, were dedicated to immunohistochemistry experiments. Whole brains were sectioned at a thickness of 20µm on a cryostat and sections were stored at 4° C in PBS until the immunohistochemistry experiments. In order to verify correct cannula placement, sections were examined to see if cannulae terminated in the lateral ventricles. Animals whose cannulae did not terminate in the lateral ventricle were removed from the analyses. Sections were placed in plastic wells and remained free floating until the completion of the immunohistochemistry procedure. Sections were initially blocked for 45 minutes in a blocking solution containing 5% normal goat serum (NGS), 0.05% Tween, and PBS. Sections were then incubated overnight at room temperature in primary antibody solution containing 5% NGS, 0.3% Tween, 0.05% sodium azide, and primary polyclonal antibody raised in rabbit directed against the VAChT (1:750 dilution). All wells were then rinsed five times for five minutes each in an excess of wash solution (PBS, 0.2% NGS, and 0.05% Tween) with gentle rotation. Following the washes, sections underwent a procedure for labeling with 3, 3' diaminobenzidine (DAB). Sections were incubated for 45 minutes in a biotinylated secondary antibody solution containing 5% NGS, PBS, and biotinylated secondary antibody (1:500 dilution; Elite Vectastain ABC kit, Burlingame, CA). Following five washes, sections were washed for 15 minutes in a hydrogen peroxide solution (3% hydrogen peroxide, 1% sodium azide, and wash solution) to eliminate non-specific binding and then washed three more times. Sections were then incubated for 45 minutes with ABC reaction in order to amplify the signal (Elite Vectastain ABC kit, Burlingame, CA) followed by another five washes. Finally, syringe-filtered DAB was

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placed into the wells while the sections were free-floating for four minutes and then washed away using wash solution. Sections were then mounted onto slides for imaging under the microscope.

Cell Counting

To determine the extent of cholinergic neuron loss in subjects from all groups administered the AF64A or sham lesion, slides were examined at an objective of 10X or 20X using a Zeiss Axioskop II Plus microscope (Carl Zeiss MicroImaging, Inc, Thornwood, NY). Cell counts of VAChT-positive neurons within the basal forebrain and hippocampus were performed in a consistent manner across sections and groups. A minimum of six sections per animal with three to four animals per group was utilized to determine relative cholinergic abundance expressed as a percent of the sham controls.

Statistical Analysis

Hidden platform training data were analyzed using the SPSS statistical software package by a repeated measures analysis of variance (ANOVA). Visible platform training and probe trial data were analyzed by one-way ANOVA. Western Blot densitometry and cell count data were also analyzed by one-way ANOVA. Tukey post-hoc comparisons of treatment groups were performed following a significant ANOVA.

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CHAPTER 4

RESULTS

Experiment 1

Morris Water Maze

An analysis of each group's latency to reach the hidden platform in the Morris water maze revealed no significant differences among the groups ($F_{3,124} = 0.208$, p=.891; see figure 1A). Similarly, there were no significant differences among the groups in latency to find the visible platform ($F_{3,131} = 1.197$, p=0.314; see figure 1A). Speed of swimming was analyzed to investigate any differences in motoric ability among the groups. No significant differences were found among the groups with regard to swim speed during hidden training ($F_{3,124} = 0.975$, p=.407; see figure 1B) or visible training ($F_{3,131} = 1.731$, p=0.164; see figure 1B). Thigmotaxis, a measurement of how much time subjects spent around the outer perimeter of the maze, is typically used as a measure of anxiety. During hidden platform training, no significant differences were observed among the groups in thigmotaxis ($F_{3,124} = 0.173$, p=.915; see figure 1C). However, during visible platform training, significant differences were observed in thigmotaxis ($F_{3,131} = 3.114$, $p<.05$; Tukey post-hocs revealed that the donepezil-galanin group had significantly higher thigmotaxis than the saline-ACSF group, $p<.05$; see figure 1C).

Following hidden platform training, the platform was removed and a probe trial was conducted to assess spatial learning (see figure 1D). A lack of a significant probe trial indicates a group did not learn the spatial location of the platform. Subjects in the saline-ACSF group spent significantly more time in the target quadrant versus each of the three non-target quadrants indicating a selective preference for the target location ($F_{3,32} =$

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27.912, p<.01; Tukey post-hoc comparisons of target quadrant versus all non-target quadrants, p<.01). Rats administered donepezil and ACSF also displayed a significant search ($F_{3,24} = 11.834$, p<.01; Tukey post-hoc comparisons of target versus all non-target quadrants, p<.01). Animals that received saline and galanin did not show a selective search as not all non-target quadrants were significant versus the target ($F_{3,28} = 5.759$, p<.01; Tukey post-hocs revealed target was only significant versus quadrant 1, p<.01, and non-significant versus quadrants 2 and 3, p>.05) which is consistent with previous studies with galanin. Interestingly, in the group given donepezil and galanin, rats did not show a preference for the target quadrant ($F_{3,28} = 2.350$, p=.094).

Experiment 2

Morris Water Maze

Administration of the cholinergic neurotoxin alone or in combination with galanin and/or donepezil did not affect the animals' latencies to find the hidden platform in the Morris water maze as is evidenced by the observation of no significant differences among the groups ($F_{4,167} = 0.555$, p=.696; see figure 2A). Interestingly, during visible platform training there was a significant difference in latency to reach the platform ($F_{4,157} = 2.769$, p<.05; Tukey post-hocs revealed that the lesion plus donepezil and galanin led to a significantly higher latency to reach the visible platform as compared to sham controls, p<.05; see figure 2A). No swim speed differences were observed in either hidden training ($F_{4,167} = 2.181$, p=.073) or visible platform training ($F_{4,157} = 0.521$, p=.721) among the groups (see figure 2B). When thigmotaxis was analyzed, a significant difference was found among the groups during hidden platform training ($F_{4,167} = 2.760$, p<.05; Tukey post-hocs showed the lesion plus donepezil and galanin produced significantly higher

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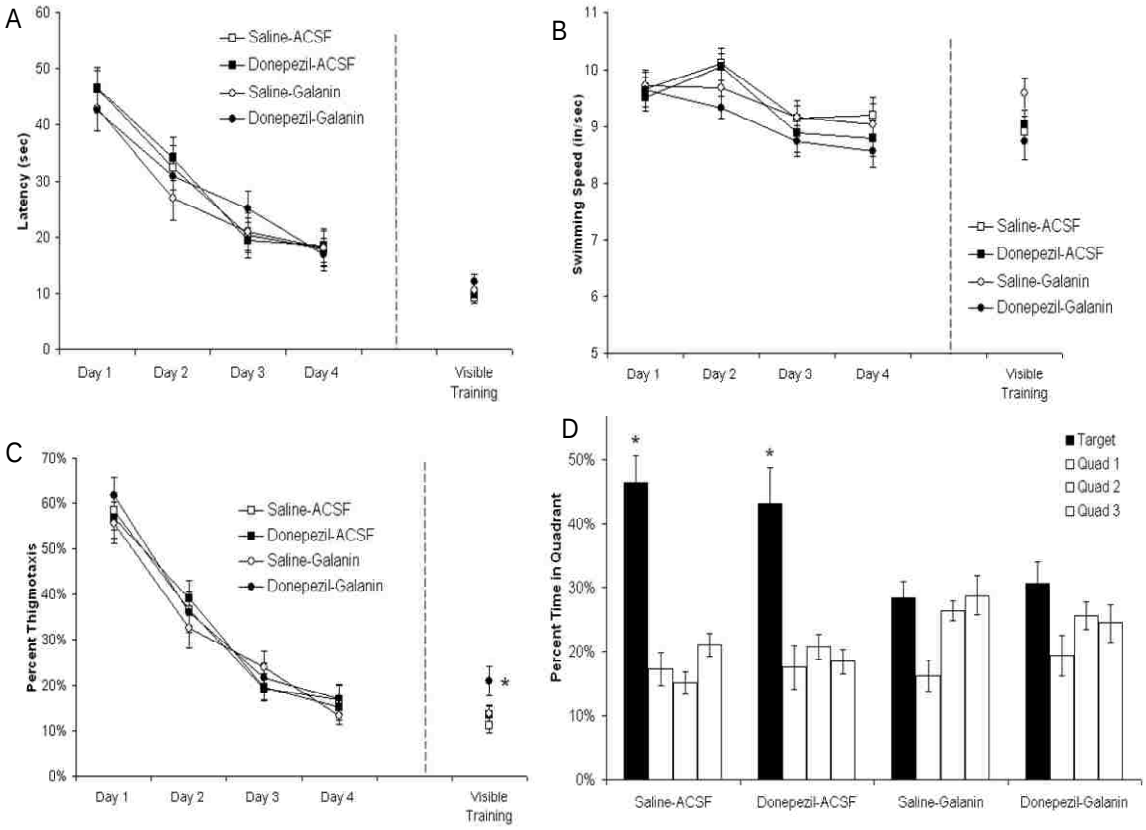

Figure 1. Water maze performance in experiment 1 following donepezil and/or galanin administration. (A) Latency for hidden and visible platform training. No significant differences were found among treatments during either condition ($p>.05$). (B) No swim speed differences were detected among groups in either hidden or visible platform training ($p>.05$). (C) An examination of time spent around the outer perimeter of the maze revealed a significant difference in thigmotaxis during visible platform training in the donepezil-galanin group ($p<.05$) versus controls but no differences during hidden training. (D) Probe trial data show that only saline controls ($p<.01$) and donepezil-administered animals ($p<.01$) spent significantly more time in the target quadrant versus each non-target quadrant indicative of a selective search. Groups administered galanin or donepezil and galanin did not display a selective search ($p>.05$).

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thigmotaxis as compared to the sham controls, $p<.05$; see figure 2C). Similarly, during visible platform training significant differences were found among treatments ($F_{4,157} = 3.603$, $p<.01$; Tukey post-hocs showed no significant differences compared to sham controls; however, the lesion plus donepezil and galanin produced significantly higher thigmotaxis as compared to the lesion group, the lesion plus donepezil, and the lesion plus galanin, $ps<.05$; see figure 2C).

Figure 2D depicts probe trial performance among the groups administered either AF64A or a sham lesion. Sham controls demonstrated a selective search as is evidenced by their significant probe trial ($F_{3,32} = 59.444$, $p<.01$; Tukey post-hoc comparisons of target versus all non-target quadrants, $p<.01$). Animals administered the lesion alone also showed a selective probe trial ($F_{3,32} = 13.231$, $p<.01$; Tukey post-hoc comparisons of target versus all non-target quadrants, $p<.01$). Surprisingly, rats infused with galanin following the lesion demonstrated a significant search as well ($F_{3,28} = 38.027$, $p<.01$; Tukey post-hoc comparisons of target versus all non-target quadrants, $p<.01$). Equally surprising was that the lesion plus donepezil and galanin group also exhibited a significant search on the probe trial ($F_{3,32} = 44.361$, $p<.01$; Tukey post-hoc comparisons of target versus non-target quadrants, $p<.01$). Interestingly, the group administered donepezil and ACSF following the lesion did not display a selective search during the probe trial ($F_{3,28} = 5.345$, $p<.01$; Tukey post-hocs revealed target was only significant versus quadrant 3, $p<.01$, and non-significant versus quadrants 1 and 2, $p>.05$).

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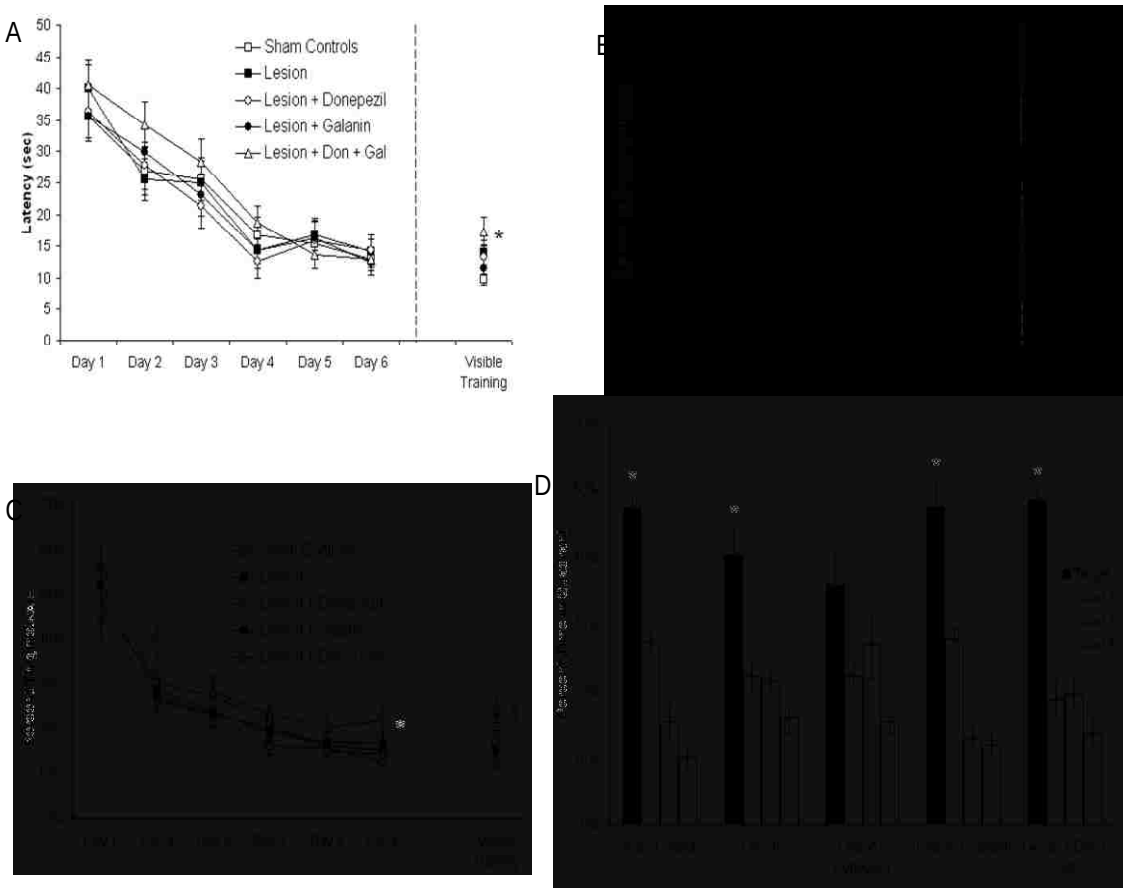

Figure 2. Water maze performance in experiment 2 following a lesion and combinations of donepezil and galanin. (A) No differences were observed during hidden platform training although the lesion plus donepezil and galanin group displayed a significantly increased latency to find the visible platform ($p<.05$) versus sham controls. (B) No significant differences in swim speed were observed in either hidden or visible platform training ($p>.05$). (C) A significantly increased thigmotaxis was observed in the lesion plus donepezil and galanin group during hidden platform training ($p<.05$) versus sham controls and during visible platform training ($p<.05$) versus lesion, lesion plus donepezil, and lesion plus galanin groups but not sham controls. (D) Probe trial data indicate that sham controls ($p<.01$), lesion ($p<.01$), lesion plus galanin ($p<.01$), and lesion plus donepezil and galanin ($p<.01$) groups all spent significantly more time in the target quadrant versus each non-target quadrant. However, animals administered the lesion plus donepezil did not display a selective search indicating they did not learn the location of the platform (analysis of target versus non-target quadrants, $p>.05$).

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Western Blotting

Western Blots were analyzed for image intensity and statistics were performed to examine differences among treatments. Representative blots of frontal cortex are shown in figure 3A. While there are indications of differences among groups, no significant differences among treatments were observed in protein levels of VAChT in the cortex ($F_{4,31} = 1.637$, p=.190; see figure 3A). A representative blot for the hippocampus is shown in figure 3B. Similar to cortex samples, although differences exist among treatments, no significant differences were found among groups in the hippocampus ($F_{4,35} = 1.435$, p=.243; see figure 3B).

Cell Counts

Cell counts performed under light microscopy of DAB-stained tissue were done in the left and right basal forebrain region and left and right hippocampi to assess the number of cholinergic neurons. A significant difference was observed among the groups in the total number of VAChT-positive cells counted in the left and right basal forebrain ($F_{4,203} = 3.464$, p<.01; Tukey post-hocs revealed that the lesion plus galanin group had significantly fewer cholinergic-positive neurons in the basal forebrain as compared to sham controls, p<.05; see figure 4D). The lesion alone group displayed a non-significant trend toward a loss of cholinergic cells as compared to sham controls, however it did not reach significance, p=.095. Representative images of basal forebrain for sham controls, lesion, and lesion plus galanin groups are depicted in figure 4 (A, B, and C, respectively). Images for lesion plus donepezil and lesion plus donepezil and galanin groups are not shown due to the lack of significant differences between these groups and controls. Cell

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counts performed in the hippocampus revealed no significant differences among treatments ($F_{4,74} = 0.613$, p=.655; data not shown).

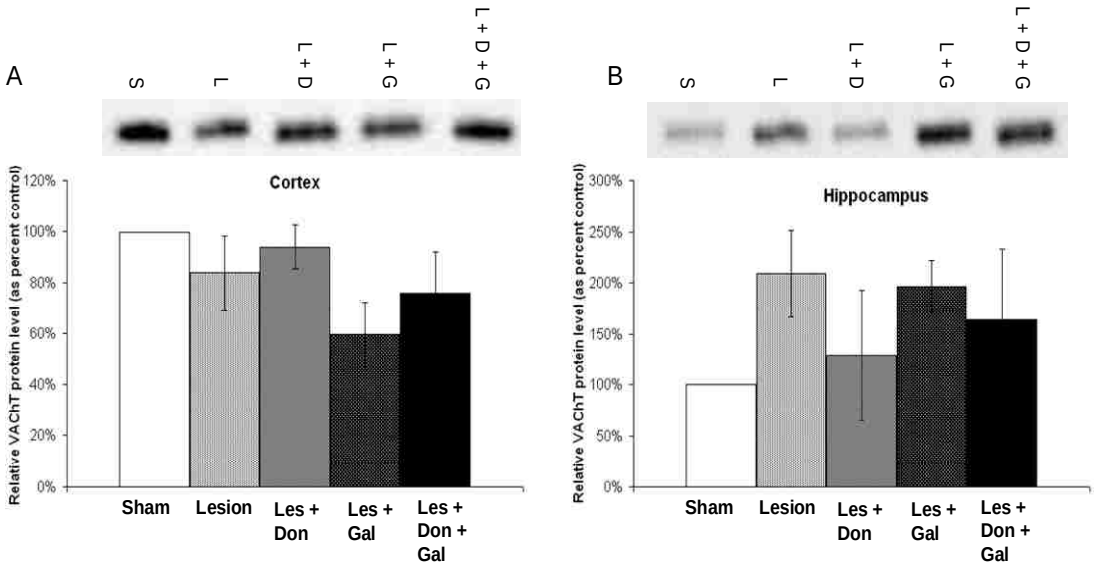

Figure 3. Western Blot analyses. (A) Representative blots of samples from the frontal cortex along with the relative VAChT protein levels in each group compared to sham controls. Analyses of Western Blot densitometry revealed no significant differences among the groups (p>.05). (B) Representative blots of hippocampal samples along with the relative protein levels of VAChT compared to sham controls. No significant differences were observed among the groups (p>.05). S: sham controls; L: lesion; L + D: lesion + donepezil; L + G: lesion + galanin; L + D + G: lesion + donepezil + galanin.

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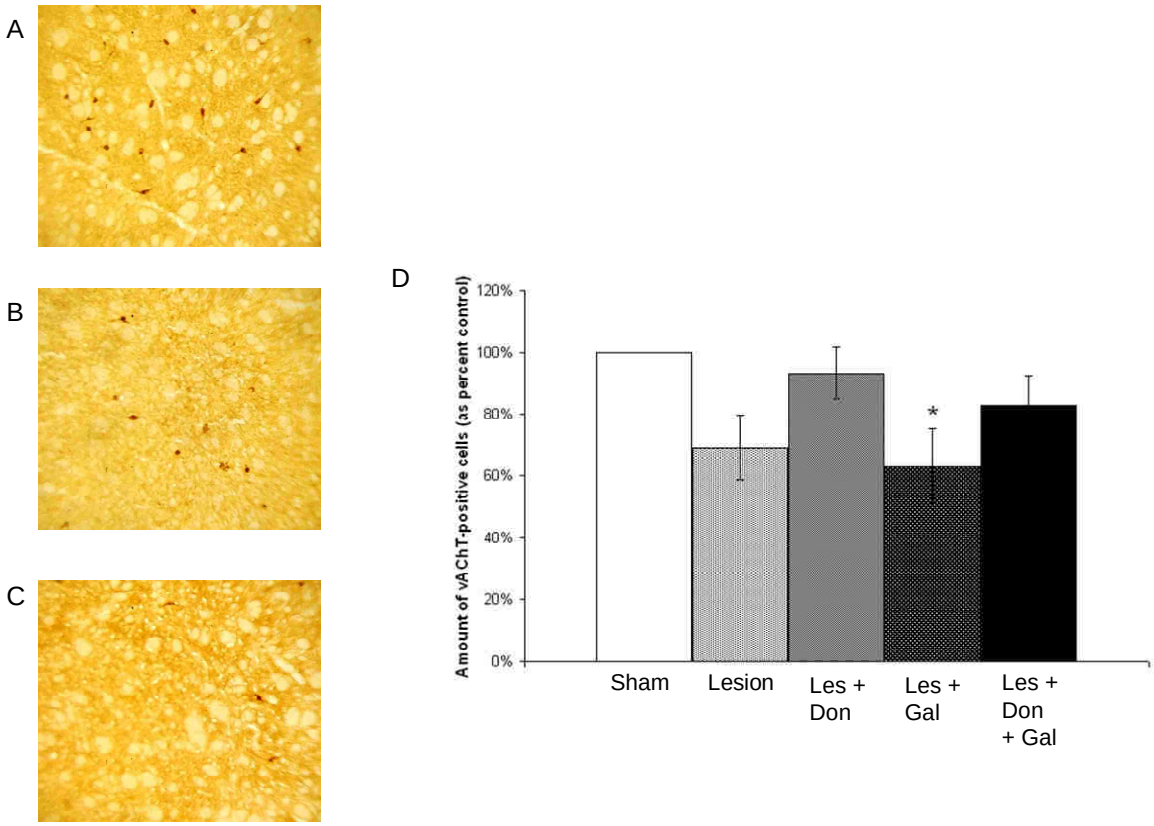

Figure 4. Immunohistochemical cell counts within DAB-stained basal forebrain sections. (A) Representative image of a sham control brain. (B) Representative image of a lesion animal. (C) Representative image of a rat from the lesion plus galanin group. (D) An analysis of cell counts performed within the basal forebrain revealed that the lesion plus galanin group had significantly fewer VAChT-positive cells than sham controls ($p<.01$). All images were obtained at a 10X magnification.

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CHAPTER 5

SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

Discussion of Results

In the above studies we investigated the interaction of galanin and the cholinergic system with particular relevance to AD. Here we present the first behavioral evidence that the galanin-induced learning and memory impairment is likely not related to galanin modulating cholinergic signaling. Experiment 1 demonstrated that the previously observed deficit in spatial learning and memory due to galanin was not rescued or even modified by the administration of the AChE inhibitor donepezil. Several previous investigations have made a case for the impairments produced by galanin being driven by altering cholinergic tone (Melander et al., 1985; Fisone et al., 1987; Dutar et al., 1989; Mufson et al., 1998). This connection has also been used extensively to support the models of galanin overexpression in AD. If the galanin-induced alteration of cholinergic signaling were in part responsible for its learning and memory deficits, the increase in cholinergic tone produced by donepezil should have altered and/or rescued the galanin-induced impairment.

In a recent study investigating the mechanisms responsible for the galanin learning deficit, it was found that galanin significantly reduced CREB phosphorylation (Kinney et al., 2009). Further, another investigation found that the administration of forskolin (an AC activator) was able to rescue the galanin-induced deficit (Kinney et. al. 2003), providing further support that the spatial learning and memory deficits due to galanin may be mediated by its inhibition of AC activity and downstream phosphorylation of CREB. Taken together, these data suggest that the investigation of

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galanin deficits should focus more specifically on the intracellular signaling cascades modulated by galanin. However, the possibility still remains that the cholinergic cell loss and overexpression of galanin observed in AD result in a completely different scenario.

In order to examine the relationship between cholinergic cell loss similar to what is observed in AD and increased galanin levels, a cholinergic neurotoxin was centrally administered in experiment 2. When the interactions of donepezil and galanin were examined in a compromised cholinergic system, a completely different behavioral profile emerged. Most notably, the galanin-induced deficit, which has been well characterized in tasks of spatial learning and memory, was absent following the cholinergic lesion. In addition, donepezil administration following the lesion produced a deficit in the probe trial indicating these animals did not learn the spatial location of the platform. This result was somewhat surprising as the majority of the literature suggests that increasing cholinergic tone is beneficial to an organism. Importantly, co-administration of donepezil and galanin following the cholinergic lesion did not produce a learning and memory impairment even though higher thigmotaxis was seen throughout training in this group. Further, a deficit in latency to reach the visible platform was observed in these animals which can largely be accounted for by the increase in thigmotaxis observed during visible training. Because this group did not show any impairment in hidden platform training or the probe trial, this visible platform deficit is not likely due to any visuomotor impairments. Interestingly, animals co-administered donepezil and galanin in experiment 1 also displayed increased thigmotaxis during visible platform training suggesting an anxiety phenotype may be present following co-administration of these compounds.

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Future studies may be able to clarify any anxiogenic effects related to the interaction of galanin and donepezil.

In the group administered a cholinergic lesion followed by saline and ACSF, no spatial learning and memory deficit was detected. Although this result was also unexpected, the data on spatial learning and memory following a cholinergic lesion are fairly inconsistent (Walsh et al., 1984; Lamberty et al., 1992; Nakamura et al., 1992; Opello et al., 1993; Dornan et al., 1996; Bizon et al., 2003; Frick et al., 2004; Dashniani et al., 2009). Whether or not a learning deficit is observed is likely tied to the extent of the cholinergic lesion and the particular neurotoxin employed. The unilateral cholinergic lesion administered in the current study was selected to induce mild damage to the basal forebrain, in an effort to mimic early or even preclinical AD. Thus, because our cholinergic insult was relatively small, the spatial learning task utilized may not have been sensitive enough to detect any behavioral changes due to the lesion. However, it is clear the lesion itself did have a physiological and behavioral impact as the effects of galanin and donepezil become radically different following the lesion versus in a normal, non-lesioned animal. Further, although not significant, there was a marked reduction in the amount of cholinergic-positive cells in the basal forebrain region of lesioned animals.

The results from the Western Blot and immunohistochemistry (IHC) experiments are a little less clear. Although disagreements exist on the accuracy of quantitative IHC techniques, the IHC experiments disclose useful and interesting information regarding neurobiological changes. Based on our data, there is a significant reduction in the amount of VAChT-positive cells in the basal forebrain region in animals administered the lesion plus galanin as compared to sham controls. This finding is contrary to what we expected

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given the trophic effects that have been observed following galanin administration *in vitro* (Zini et al., 1993; Holmes et al., 2000; O'Meara et al., 2000; Mahoney et al., 2003; Elliot-Hunt et al., 2007). Further, because galanin administration following the lesion did not produce a learning and memory deficit in the water maze, this significant reduction in cholinergic-positive cells is even more surprising. The possibility exists that although galanin may not exhibit any neuroprotective qualities *in vivo*, its effects in a compromised cholinergic system are different than in a normal, non-lesioned animal. More data related to the *in vivo* effects of galanin in animal models relevant to the specific disease state may be necessary to clarify this galanin-induced change.

There is also a non-significant trend (p=.095) towards a reduction in cholinergic cells in lesioned animals. Although this reduction did not reach significance in this group, it is clear the lesion did alter the amount of VAChT-positive cells in the basal forebrain. Interestingly, in the lesion plus donepezil and lesion plus donepezil and galanin groups, there is very little change in the amount of cholinergic neurons as compared to sham controls. Whether this lack of a reduction in cholinergic cells in these groups reflects any neuroprotective properties of donepezil is difficult to determine with the data at hand. However, previous studies have discovered possible neuroprotective effects of donepezil (Takada-Takatori et al., 2006; Akasofu et al., 2008), raising the possibility that daily donepezil administration may have negated the effects of the lesion in the current study. Interestingly, impairments in water maze learning were observed in the group administered donepezil following the lesion. These results suggest compensatory changes following the lesion in combination with donepezil administration may have disrupted

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spatial memory. Further possibilities regarding the behavioral deficit in this group are outlined below.

An analysis of the densitometry of the Western blots revealed no significant differences among the groups in either the cortex or the hippocampus. Interestingly however, the trends in the VAChT protein levels in the cortex seem to mirror the group differences observed following the IHC cell counts. The consistency observed between procedures in the differences among groups reinforces the validity of the findings. Further, although there is a large amount of variability within each group, clear trends are observable in the hippocampus as well. It is interesting to note that these trends tend to be opposed to the data from the Western blot cortex samples and the IHC basal forebrain cell counts. Spatial learning and memory in the Morris water maze is a hippocampally-dependent task. Therefore, the shift in VAChT protein levels in the hippocampus in some of these groups could potentially account for why a deficit is observed in the lesion plus donepezil group while none is found in the lesion plus galanin group. Specifically, the Western data indicate that the lesion plus donepezil group has lower levels of VAChT protein in the hippocampus than the lesion and lesion plus galanin groups. This difference may play a role in the lesion plus donepezil group deficit, as further outlined below. The large variability observed within each group precludes the discovery of any significant differences among these groups, and a replication of these findings may be necessary before any definitive conclusions can be reached regarding the neurobiological effects of donepezil and/or galanin following a lesion.

The data from the above experiments indicate an interesting shift in the effects of galanin as well as the interaction of donepezil and galanin. In experiment 1, donepezil

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alone did not alter performance while galanin impaired spatial learning and memory consistent with previous investigations. Interestingly, the galanin deficit was still present when donepezil was co-administered indicating the galanin learning impairment is likely not related to the suppression of cholinergic signaling. However, in the presence of a cholinergic lesion these effects were inverted. Following perturbation of the cholinergic system as in AD, the same dose of donepezil produced a spatial learning impairment while the same concentration of galanin (from the same batch) or co-administration of galanin and donepezil did not lead to a learning impairment. Even more compelling is that the cholinergic lesion alone was insufficient to impair learning and memory in the water maze. These data suggest that there are differential effects of both galanin and donepezil depending on the level of cholinergic tone. Further, it is also interesting to note that while the galanin deficit does not appear to be tied directly to the modulation of ACh, following the reduction of cholinergic tone as in AD, galanin does not produce an impairment. In addition, although administration of donepezil following the lesion impaired water maze performance, when galanin was co-administered with donepezil, this donepezil-induced deficit vanished. These data suggest that galanin may have a beneficial role in a compromised cholinergic system similar to what is observed in AD. These findings also support a more detailed investigation of galanin activity in specific disease state models as opposed to previous investigations of exogenous galanin administered to normal subjects.

The role of galanin in AD is currently unknown with many investigators suggesting it may be exacerbating cognitive symptoms while others support a potential trophic role for galanin in the disorder. Interestingly, galanin has been shown to protect

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neurons from the toxic effects of Aβ (Ding et al., 2006; Cheng & Yu, 2010; Cui et al., 2010) and has also recently been demonstrated to rescue Aβ-induced learning and memory deficits in the Morris water maze (Cheng & Yu, 2010). These studies suggest the overexpression of galanin may serve a protective role in AD in an attempt to counteract the neurotoxic effects of Aβ. In the current study when cholinergic functioning was altered, galanin may also display protective properties similar to what is seen following Aβ infusion. However, the histological data did not reveal any neuroprotective qualities of galanin, indicating the lack of a behavioral deficit may be unrelated to galanin's trophic properties.

The cholinergic lesion also altered the behavioral outcome of donepezil administration, producing a deficit where none was observed without the lesion. This finding suggests neurological changes due to the lesion, perhaps compensatory in nature, followed by the inhibition of AChE may have created a scenario where the increase in cholinergic tone impaired spatial learning. In fact, there are reports that also demonstrate spatial learning deficits due to increasing cholinergic tone (Bunce et al., 2004; Elvander et al., 2004; Sabolek et al., 2005), particularly in the retrieval of previously learned information (Rogers & Kesner, 2004). This retrieval deficit is especially relevant in our study considering donepezil administration following the cholinergic lesion impaired probe trial performance in the Morris water maze. However, further studies are necessary to confirm and elucidate the mechanisms driving this donepezil-induced change. In addition, no deficit was observed following co-administration of donepezil and galanin in rats with a compromised cholinergic system, suggesting whatever effect galanin has following a lesion is sufficient to rescue the donepezil-induced deficit. It is possible that

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the increase in cholinergic tone caused by donepezil was reduced by galanin administration (Fisone et al., 1987; Dutar et al., 1989; Palazzi et al., 1991), which restored ACh levels to a level consistent with other groups and eliminated the donepezil-induced deficit. Clearly, the interactions between galanin and the cholinergic system become more complex following a cholinergic lesion mimicking early AD and more data may be required before any definitive interpretations can be made.

Regardless of whether or not galanin plays a neuroprotective role in AD, based on the findings from experiment 1, the behavioral deficits attributed to galanin are more likely due to its inhibition of CREB phosphorylation (Kinney et al., 2009) and not tied to suppression of cholinergic signaling. Further, it may be necessary to readdress the behavioral consequences of galanin's modulation of cholinergic functioning, especially in early or preclinical AD when ACh levels begin to fall and cognitive deficits begin to arise. A potentially more relevant approach to galanin in AD may be to address the mechanisms responsible for its overexpression in AD. This approach may provide valuable data regarding the mechanisms involved in the pathology of AD. For example, several groups have argued that calcium alterations may be tied to both the neurofibrillary tangles and Aβ plaques observed in AD (McKee et al., 1990; Hardy & Higgins, 1992; Mattson et al., 1992; Murray et al., 1992; Querfurth & Selkoe, 1994; Green & Laferla, 2008; Lopez et al., 2008; Vale et al., 2010). As the transcriptional regulation of galanin is on a calcium-dependent switch, specifically a CREB-dependent transcription factor (Zachariou et al., 2001), the overexpression of galanin may be tied to other pathological findings in AD. Changes in calcium levels consistently observed in AD (Peterson et al., 1985; Landfield et al., 1989; Thibault & Landfield, 1996; Raza et al.,

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2007; Thibault et al., 2007; Bezprozvanny and Mattson, 2008; Bojarski et al., 2008) could potentially lead to increased CREB activation and an upregulation of galanin. In addition, as galanin has demonstrated neuroprotection against calcium-induced damage (Palazzi et al., 1991; Mazarati et al., 2000; Arabadzisz et al., 2005; Mazarati et al., 2006; Endoh et al., 2008), these changes in calcium levels alone could lead to the overexpression of galanin in AD.

The potential upregulation of galanin following calcium dysegulation raises the possibility of utilizing galanin as a marker in animal models and perhaps AD as well. If the overexpression of galanin observed in AD and animal models of AD (Baraka & ElGhotny, 2010) is due to changes in calcium levels, then measuring levels of galanin may prove fruitful in the detection of AD *in vivo*. The development of galanin receptor ligands for clinical use has proven challenging. Therefore, investigations of galanin in AD may be better served by examining levels of galanin as a marker for cell damage or cell loss due to calcium alterations.

The role of galanin in inhibiting the evoked release of classical neurotransmitters and preventing neuronal hyperactivity has been well documented (Dutar et al., 1989; Pieribone et al., 1995; Kinney et al., 1998; Xu et al., 1998). However, galanin's inhibition of CREB phosphorylation provides a possible alternative explanation for its potential neuroprotective properties in a damaged nervous system. By inhibiting AC and downstream phosphorylation of CREB, galanin may be conserving vital resources by halting the activation of several transcription factors. This reduction in transcription may allow a struggling nervous system to allocate resources where necessary without expending unwanted energy, which could be critical in a neurodegenerative disorder such

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as AD. In the current study, galanin did not produce a spatial learning and memory impairment when administered to an animal with a compromised cholinergic system. This finding is unique as previous investigations with several learning and memory tasks have typically demonstrated learning impairments following galanin administration. Together with results from previous studies indicating galanin inhibits CREB phosphorylation (Kinney et al., 2009), these findings could potentially support a role for galanin in the preservation of a damaged nervous system in response to cellular insults as may be occurring in AD.

The cognitive effects of galanin overexpression in AD are currently unknown while many investigators suggest galanin may be exacerbating cognitive deficits by suppressing cholinergic tone. The current study emphasizes that the spatial learning and memory impairments following galanin administration are likely distinct from cholinergic modulation. Previous work indicates perhaps more attention instead should be paid to galanin's inhibition of CREB phosphorylation as a potential modulator of learning and memory (Kinney et al., 2003; Kinney et al., 2009). Further, the data suggest galanin administration may be beneficial for spatial learning in an animal model of AD mimicking cholinergic loss as well as in other studies following infusion of Aβ peptide (Cheng & Yu, 2010). Although more studies are necessary to elucidate any neuroprotective effects of galanin *in vivo*, our data argue for a reappraisal of the role of galanin in learning and memory as well as AD. These findings may also impact the utility of administering galanin receptor ligands both in AD as well as other neurodegenerative disorders.

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