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ALPHA7 NICOTINIC ACETYLCHOLINE RECEPTOR REGULATION IN EXPERIMENTAL NEURODEGENERATIVE DISEASE

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ABSTRACT OF DISSERTATION

Christina Margaret Charriez

The Graduate School

University of Kentucky

2010

ALPHA7 NICOTINIC ACETYLCHOLINE RECEPTOR REGULATION
IN EXPERIMENTAL NEURODEGENERATIVE DISEASE

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Pharmacy
at the University of Kentucky

By
Christina Margaret Charriez

Lexington, Kentucky

Director: James R. Pauly, Ph.D., Associate Professor of Pharmaceutical Sciences

Lexington, Kentucky

2010

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ABSTRACT OF DISSERTATION

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The $\alpha 7$ nicotinic acetylcholine receptor (nAChR) is involved in learning and memory, synaptic plasticity, neuroprotection, inflammation, and presynaptic regulation of neurotransmitter release. Alzheimer's disease (AD), a neurodegenerative disease characterized by diminished cognitive abilities, memory loss, and neuropsychiatric disturbances, is associated with a loss of nAChRs. Similarly, traumatic brain injury (TBI) may result in long term neurobehavioral changes exemplified by cognitive dysfunction. Deficits in $\alpha 7$ nAChR expression have previously been shown in experimental TBI and may be related to cognitive impairment experienced in patients following TBI.

The purpose of this dissertation was to investigate changes in $\alpha 7$ nAChR expression in models of neurodegeneration and determine if allosteric modulation of the nAChR facilitates functional recovery following experimental TBI through changes in nAChRs. Experimental models employed include a transgenic mouse model of AD that overexpresses the amyloid precursor protein (APP^{swe} mice) and the controlled cortical impact injury model of TBI in rats. Quantitative receptor autoradiography using α -[¹²⁵I]-bungarotoxin and [¹²⁵I]-epibatidine and *in situ* hybridization were used to investigate changes in nAChR density and mRNA expression, respectively.

In the first study, the effects of aging and β -amyloid on $\alpha 7$ nAChR expression were evaluated in APP^{swe} mice. Hippocampal $\alpha 7$ nAChR density was significantly upregulated in APP^{swe} mice compared to wild-type mice. It is postulated that elevated A β levels bind to the $\alpha 7$ nAChR resulting in upregulation. In a second study, galantamine, a medication used in the treatment of AD, was administered subchronically following experimental TBI to determine if treatment could facilitate cognitive recovery and affect nAChR expression. Interestingly, the results indicate TBI interferes with agonist mediated upregulation of nAChRs, and galantamine did not improve function in a behavioral task of learning a memory. In a third study, the regulation of TBI related deficits in $\alpha 7$ nAChRs was examined 48 hours following injury. $\alpha 7$ nAChR deficits occurred with a reduction in $\alpha 7$ mRNA in several hippocampal regions and non- $\alpha 7$ nAChR deficits occurred with a reduction in $\alpha 4$ mRNA in the metathalamus. The results

of these studies suggest AD and TBI may involve complex but parallel processes contributing to the regulation of $\alpha 7$ nAChRs.

KEYWORDS: Nicotinic Acetylcholine Receptor, Alzheimer's Disease, Traumatic Brain Injury, Beta-amyloid, Galantamine

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August 3, 2010

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DISSERTATION

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The Graduate School

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Chapter 1: Introduction to Alzheimer's Disease and Traumatic Brain Injury; Statement of the Research Problem and Plan

Neuronal Nicotinic Receptors

Neuronal nicotinic acetylcholine receptors (nAChRs) are the focus of extensive research due to their involvement in numerous important physiological processes such as cognitive learning and memory, synaptic plasticity, and neuroprotection (Levin and Simon, 1998; Paterson and Nordberg, 2000; Levin et al., 2002). Furthermore, nAChRs are involved in arousal, cerebral blood flow and metabolism, inflammation, and presynaptic regulation of neurotransmitter release, and nAChR expression is altered in several pathophysiological conditions. Nicotinic acetylcholine receptors (nAChRs) are one of two classes of receptors involved in cholinergic neurotransmission in the central nervous system (CNS) (Cooper et al., 2003). Cholinergic neurotransmission first involves the production of the neurochemical mediator, acetylcholine (ACh), in presynaptic cholinergic neurons by the synthetic activity of choline acetyltransferase (ChAT). For this process, an acetyl group from mitochondrial-derived acetyl-coenzyme A is transferred to choline, a dietary nutrient, which undergoes uptake into the presynaptic neuron by the rate-limiting, sodium dependent, high-affinity choline uptake (HACU). ACh is stored in vesicles located near the synaptic terminal and upon depolarization, calcium dependent release occurs. Synaptic ACh may then bind to postsynaptic ACh receptors, or may regulate its own release via interactions with presynaptic receptors. Unbound ACh in the synapse is hydrolyzed by acetylcholinesterase (AChE) present on pre- and postsynaptic cell membranes. Following activation of postsynaptic receptors, ACh is released from receptors and degraded by AChE to acetate and choline, which is then recycled in the synthetic process.

Major CNS cholinergic pathways in the rodent brain include two projections (Butcher and Woolf, 1986; Woolf and Butcher, 1989; Cooper et al., 2003). The first of which arises from the basal forebrain which includes the medial septal nucleus, diagonal band nuclei, substantia innominata, magnocellular preoptic field area, and nucleus basalis projecting to the neocortex. The second arises from pedunclopontine and laterodorsal

tegmental nuclei and projects to the thalamus and other diencephalic loci, pontine and medullary reticular formations, deep cerebellar and vestibular nuclei, and cranial nerve nuclei. There are also cholinergic interneurons (short-projections) located in regions of the basal ganglia (caudate-putamen nucleus, nucleus accumbens), the olfactory tubercle, and the granule cells therein called the Islands of Calleja. Human pathways are similar and are outlined in detail elsewhere (Arciniegas, 2003; Salmond et al., 2005).

The neurotransmitter ACh interacts with two distinct cholinergic receptor subtypes, nicotinic and muscarinic (Cooper et al., 2003). While muscarinic acetylcholine receptors (mAChRs) are metabotropic receptors coupled to G-proteins (guanine nucleotide binding regulatory proteins) eliciting inhibitory or excitatory responses, nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels in which activation results in increased cellular sodium and calcium permeability as well as depolarization and excitation. Muscarinic receptors were first discovered through the agonistic properties of the mushroom toxin, muscarine, within the parasympathetic nervous system (Albuquerque et al., 2009). Muscarinic receptors exist as five subtypes designated as M1-M5. Muscarinic M1, M3, and M5 subtypes bind to the G_q/G_{11} type of G-proteins and modulate phosphatidylinositol signaling, whereas M2 and M4 bind to the G_i type and are mainly inhibitory acting through ion channels and cyclic adenosine monophosphate (cAMP) (Cooper et al., 2003). Receptor and subsequent G-protein activation in turn has various effects on signal transduction pathways within the cell.

Neuronal nAChRs are made up of five glycosylated polypeptide chain subunits known as α and β unlike excitatory muscular nAChRs that contain postsynaptic heteropentamers containing γ and δ (or ϵ in the fetal brain) subunits in addition to α and β (Cooper et al., 2003). The discovery of nicotinic receptors began with the original description of the stimulatory effect of the tobacco plant alkaloid, nicotine, on autonomic ganglia (Langley and Dickinson, 1889; Taylor, 1996; Albuquerque et al., 2009). The nAChR was further characterized through studies of the electric ray fish, containing high levels of nAChRs and the ability to impair prey with an electric pulse and studies of the venom of the krait snake, containing α -bungarotoxin, which induces paralysis (Albuquerque et al., 2009).

Brain nAChRs are comprised of $\alpha 2$ - $\alpha 10$ and $\beta 2$ - $\beta 4$ subunits. These subunits typically assemble in a $2\alpha 3\beta$ stoichiometry (Conroy et al., 1992) or as a single subtype homopentamer consisting of $\alpha 7$, $\alpha 8$, or $\alpha 9$ subunits (Couturier et al., 1990) that span the membrane and surround a central pore or ion channel (Cartaud et al., 1973; Paterson and Nordberg, 2000). Detailed reviews of nicotinic receptors can be found in the following references (Changeux et al., 1998; Paterson and Nordberg, 2000; Albuquerque et al., 2009). Two major classes of nAChRs exist in the CNS referred to as α -bungarotoxin (BTX), derived from a snake venom toxin that blocks muscular nAChRs, sensitive and BTX insensitive designated as $\alpha 7$ and non- $\alpha 7$, respectively (Marks and Collins, 1982; Paterson and Nordberg, 2000). The most common non- $\alpha 7$ nAChR found in mammalian CNS is the $\alpha 4\beta 2$ nAChR (Paterson and Nordberg, 2000). The $\alpha 4\beta 2$ subtype is also described as the high-affinity [^3H]-nicotine binding site, and the $\alpha 7$ subtype is referred to as the high-affinity [^{125}I]-BTX binding site (Clarke et al., 1985; Harfstrand et al., 1988; Changeux et al., 1998). Two ligand binding sites are present in heteropentamers like the $\alpha 4\beta 2$ subtype and five binding sites exist on homopentamers such as in the $\alpha 7$ subtype (Alkondon and Albuquerque, 1993; Wang et al., 1996; Paterson and Nordberg, 2000; Albuquerque et al., 2009). The binding site is located on the α subunit at the interface between α and β in the $\alpha 4\beta 2$ subtype and at the interface between two α subunits for the case of the $\alpha 7$ subtype. Two sites must be occupied to activate the channel in the $\alpha 4\beta 2$ subtype. In addition, an allosteric binding site is located on the α subunit upon which binding modulates channel opening and ion conductance (Pereira et al., 1993).

Nicotinic receptors are located pre- or postsynaptically as well as on sites outside the synapse and act to regulate neuronal function (Lindstrom, 1997). Presynaptic nAChRs are involved in regulating the release of neurotransmitters to modify other neurochemical systems. For example, primarily non- $\alpha 7$ nAChRs modulate the release of striatal dopamine and hippocampal norepinephrine, and glutamate release may be modulated by the $\alpha 7$ nAChR (Wonnacott et al., 1990; Dajas-Bailador and Wonnacott, 2004; Wonnacott et al., 2006). Neuronal nicotinic receptors exist in four conformational states including a resting state, a low-affinity ligand activated state, and two desensitized closed channel states, which are nevertheless associated with high-affinity ligand binding

(Katz and Thesleff, 1957; Galzi and Changeux, 1995). Nicotinic receptors undergo a time and concentration dependent desensitization (Katz and Thesleff, 1957; Wang and Sun, 2005) which is a decrease in response to nicotine or other agonists upon increasing exposure time. In contrast to traditional pharmacological adaptations in which repeated agonist exposure results in a compensatory decrease in receptor density, the neuronal nicotinic receptor is upregulated by chronic nicotine exposure in rodents (Marks et al., 1983; Schwartz and Kellar, 1983) and in the postmortem brains of smokers (Benwell et al., 1988; Nyback et al., 1989; Wonnacott, 1990; Breese et al., 1997a). Furthermore, the well documented neuroprotective actions of nicotine are theorized by many to be the result of receptor upregulation (Jonnala and Buccafusco, 2001). Many support the view that the paradoxical upregulation caused by chronic stimulation of nAChRs is due to increases in receptor number through post-translational modifications (Wonnacott, 1990; Marks et al., 1992; Peng et al., 1994; Pauly et al., 1996; Perry et al., 1999; Gentry and Lukas, 2002). However, a recent report using heterologous expression of rat $\alpha 4\beta 2$ in a human cell line suggested changes in function rather than receptor number occurs following chronic nicotine exposure in which stabilization of the high-affinity state and subsequent increases in binding and response to binding occurs (Vallejo et al., 2005). Additionally, the functional properties of upregulated nAChRs are a topic of debate. Functional loss has been documented in nAChRs and may be a result of desensitization which occurs both as a rapid onset, quickly reversible form and a slower persistent inactivation that delays recovery (Lukas et al., 1996; Gentry and Lukas, 2002). However, loss of function may not be associated with upregulation (Gentry and Lukas, 2002), and other studies, in particular electrophysiological analyses, have concluded that increased nAChRs are functional (Albuquerque et al., 2009).

Pharmacologic tools for studying cholinergic transmission *in vivo* involve the evaluation of presynaptic markers such as ChAT and AChE activity, ACh synthesis and HACU in human and animal tissues. Postsynaptic receptor expression is studied with immunohistochemical techniques using antibodies against the receptor protein or by the use of autoradiography, which employs a selective radioligand to measure receptor density and/or affinity in specific brain locations using tissue slices or homogenates. The measurement of nicotinic receptors by radioligands is a method used by many of the

studies discussed in this chapter and is a method employed in the dissertation research. Additionally, *in vitro* receptor expression systems are used to address mechanistic questions. Synaptic terminal preparations known as synaptosomes and electrophysiologic techniques are often used to study neurotransmitter release and receptor function.

In the rat, $\alpha 7$ nAChRs are expressed throughout the brain with the highest concentrations of transcripts in the olfactory regions, hippocampus, amygdala, and hypothalamus, which comprise the limbic system (Seguela et al., 1993). In an autoradiographic analysis of cholinergic receptors in the mouse brain, $\alpha 7$ nAChRs were most concentrated in the hippocampus, caudate putamen, inferior and superior colliculi, hypothalamus, and hindbrain (Pauly et al., 1989). Of note, $\alpha 7$ nAChRs are unique in that they are localized in high concentrations in the hippocampus and are highly permeability to calcium (Seguela et al., 1993). Non- $\alpha 7$ nAChRs are most highly localized in thalamic nuclei, the superior colliculus, and the interpeduncular nucleus (Pauly et al., 1989). By comparison, muscarinic receptor subtypes are more evenly distributed in the CNS with the highest levels in hippocampus, cerebral cortex, and colliculi.

Alzheimer's Disease

Background

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by diminished cognitive abilities, memory loss, and neuropsychiatric disturbances. Dementia is defined by impairments in memory and cognition that interfere with the activities of daily living (Alzheimer's Association, 2009), and AD is the most common form of dementia occurring in 13 percent of individuals over the age of 65. In 2000, it was estimated that 4.5 million people suffered from AD in the United States and this figure was expected to rise to 13.2 million by the year 2050 (Hebert et al., 2003). According to a recent report, 5.3 million Americans are currently living with AD (Alzheimer's Association, 2009). In addition, numerous studies have concluded that more women develop AD because of their longer life expectancy compared to men, and the more years of education one receives, the lower the risk of AD.

Patients with Alzheimer's disease often present with loss of memory, in particular difficulties in remembering new information, in the initial stages of disease (NIA, 2005; Alzheimer's Association, 2009). AD patients develop confusion and cognitive dysfunction that results in impaired judgment, decision making, orientation, and language skills, as well as alterations in personality and behavior. Eventually, patients can no longer perform the activities of daily living. The clinical diagnosis of Alzheimer's disease includes a study of the patient's medical history, clinical presentation, and neurological and psychiatric examinations (Blennow et al., 2006). Extensive laboratory analyses are performed to determine if coexisting medical conditions are present that may cause dementia, and neuroimaging with computed tomography (CT) or magnetic resonance imaging (MRI) is used to rule out brain tumors, brain injury, or cerebrovascular disease (Cummings, 2004; Blennow et al., 2006). Diagnosis also involves use of the National Institute of Neurological and Communicative Disease and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria (McKhann et al., 1984) in order to establish a probable or possible diagnosis. However, a diagnosis of definite AD is only made with concomitant neuropathological findings at autopsy (Cummings, 2004; Blennow et al., 2006).

Synaptic loss is a defining feature in AD (Selkoe, 2002). Neuroimaging studies have shown that the medial temporal lobe including the entorhinal cortex and hippocampus undergoes early neurodegeneration in which atrophy is detectable, and as the disease progresses, the Alzheimer's brain displays pervasive cortical atrophy with ventricular enlargement due to further synaptic degeneration and neuronal death (Cummings, 2004; Mattson, 2004; Blennow et al., 2006). The pathogenesis of Alzheimer's disease includes the presence of two neuropathological hallmarks, intracellular neurofibrillary tangles made up of the hyperphosphorylated form of the microtubule protein, tau, and extracellular neuritic (amyloid) plaques made up of dystrophic neurons surrounding a dense core comprised of the β -amyloid (amyloid- β or A β) peptide (Hardy and Selkoe, 2002; Selkoe, 2005). A prevailing theory known as "the amyloid cascade hypothesis" states that Alzheimer's disease results from the increased production or reduced clearance of A β (Hardy and Selkoe, 2002; Querfurth and LaFerla, 2010).

The majority of AD patients have a sporadic type, but approximately five percent of cases are inherited, familial forms of AD, in which patients present with an earlier (as young as 30 years of age), more aggressive course (St George-Hyslop, 2000; Alzheimer's Association, 2009). Familial Alzheimer's disease (FAD) can be caused by mutations in the amyloid precursor protein (APP) or the presenilins (PS1, PS2), both resulting in altered expression of A β . One such mutation is the Swedish double mutation (K670N, M671L, often referred to as APP_{swe}) in the APP gene located on chromosome 21 (Mullan et al., 1992). APP is a glycosylated cell-surface protein 770 amino acids in its longest form (Weidemann et al., 1989; Esch et al., 1990; Oltersdorf et al., 1990; Sisodia et al., 1990). APP constitutively undergoes processing through a secretory pathway in which α -secretase cleavage within the sequence containing the A β peptide results in the secretion of a soluble extracellular domain precluding A β formation. Alternatively, via a lysosomal pathway, A β is formed predominately as the 40 amino acid peptide, A β 40, through the actions of β -secretase (also known as β -site APP-cleaving enzyme 1, BACE1) and γ -secretase (Haass et al., 1992; Blennow et al., 2006; Walsh and Selkoe, 2007; Querfurth and LaFerla, 2010). Additionally a longer peptide is formed, A β 42/43, to a lesser extent. Furthermore, mutations in APP or the presenilins, the catalytic component of γ -secretase, increase the production of A β in general or increase the larger peptide, A β 42/43, that develops into an insoluble form involved in self-aggregation, oligomerization, and plaque formation (Selkoe, 1994, 2001, 2002; Mattson, 2004; Selkoe, 2005). However, the culmination of many studies has led to the belief that soluble oligomeric A β , preceding plaque formation, is a significant mediator of neurotoxicity (Walsh and Selkoe, 2007; Tomic et al., 2009; Querfurth and LaFerla, 2010). Soluble A β is presented in further detail in the Chapter 2 discussion section.

Cholinergic dysfunction in Alzheimer's disease

Although cognitive impairment in AD is undoubtedly a multifactorial process, many studies have shown that Alzheimer's disease causes a selective degeneration of basal forebrain cholinergic neurons resulting in the loss of cholinergic innervation to the cortex and hippocampus; therefore, the cognitive impairment seen in AD patients has often been correlated with deficits in CNS cholinergic neurotransmission (Bartus et al.,

1982; Bartus, 2000). Postmortem research has shown reductions in choline acetyltransferase (ChAT) (Davies and Maloney, 1976; Flynn and Mash, 1986; Araujo et al., 1988; Aubert et al., 1992; Bierer et al., 1995; Sihver et al., 1999) and acetylcholinesterase (AChE) (Davies and Maloney, 1976; Bierer et al., 1995; Perry et al., 2000) activities in various brain regions such as the cerebral cortex, amygdala, and hippocampus. One report showed ChAT activity as well as acetylcholine (ACh) synthesis and choline uptake, all markers of presynaptic terminals, were decreased by 40 percent or more in the frontal and temporal cortical lobes (Sims et al., 1983); furthermore, vesicular ACh transport was also diminished (Sihver et al., 1999). In addition, a 75 percent neurodegeneration of the nucleus basalis of Meynert (nbM), part of the basal forebrain which contains cholinergic cell bodies projecting to the cortex, was also identified (Whitehouse et al., 1982).

Deficits in ChAT activity in AD cortex have been shown to correlate with clinical dementia (Bierer et al., 1995), and ChAT and AChE activities have been shown to decrease significantly with increasing plaque load, while ChAT activity correlated with cognitive impairment in patients with dementia (Perry et al., 1978). However, one postmortem study of elderly nursing home residents showed cholinergic markers are not associated with early AD; instead, diminished ChAT and AChE activities were associated with severe clinical dementia ratings indicative of cognitive and functional status late in the progression of disease, and ChAT was found to be correlated with neurofibrillary tangles and neuritic plaque severity (Davis et al., 1999).

In addition to changes in traditional presynaptic markers, changes in nicotinic and muscarinic receptor expression have been reported in postmortem AD tissue. Nicotinic cholinergic receptor (nAChR) expression is consistently reduced in cortical and hippocampal brain samples obtained at autopsy and is the subject of several review articles (Pauly, 1999; Court et al., 2001; Nordberg, 2001). Many studies employing immunological detection methods or radioligand binding assays to characterize nAChR expression in AD have been conducted. Table 1.1 summarizes the regional changes in nAChRs determined by these studies and compares the detection techniques used. A few specific study findings not included in the summary table are discussed below.

Several studies demonstrated overall reductions in nAChR receptor numbers assessed by the binding of high affinity radiolabeled ligands. Of interest, the binding of epibatidine (EPI) and nicotine in the cerebral cortex was downregulated in AD patients with the Swedish double mutation as well as the sporadic type (Marutle et al., 1999). One report noted that a 50 percent decrease in nicotine binding in temporal cortex was accompanied by a sparing of postsynaptic nAChRs in frontal cortex, yet diminished ChAT activity in both regions indicated loss of presynaptic terminals (Flynn and Mash, 1986). Of note, the validity of the BTX studies reviewed in the summary table should be considered in light of the data suggesting up to 70 percent of receptor binding is due to non-specific binding when autoradiography of $\alpha 7$ nAChRs in human brain tissue is employed using BTX (Breese et al., 1997b; Spurden et al., 1997; Court et al., 2001). Decreases in EPI binding in the temporal cortex correlated with worsening dementia, increased A β 42 levels, and reduced AChE activity (Perry et al., 2000). Increased A β containing plaques were not correlated with diminished EPI binding, but instead were correlated with reductions in nicotine binding in the entorhinal cortex and $\alpha 4$ protein expression in the temporal cortex. In contrast, increased BTX binding correlated with plaques in the entorhinal cortex. Positron emission tomography (PET) imaging has allowed for the assessment of nAChR densities *in vivo*. *In vivo* nicotine binding assessed by PET has demonstrated reductions in nAChR densities in cerebral cortex and hippocampus in patients with AD (Nordberg et al., 1990; Nordberg et al., 1995; Nordberg et al., 1997).

Reductions in $\alpha 4$ and $\alpha 7$ nAChR protein have been found in the hippocampus and temporal cortex of patients with both sporadic AD and patients with the FAD related Swedish double mutation (Yu et al., 2005). One study showed the reduction in $\alpha 7$ nAChR protein was accompanied by neuronal loss (Banerjee et al., 2000). A localized deficiency or near absence in cortical $\alpha 4$ and $\alpha 7$ mRNA was detected in neurons that were densely labeled with hyperphosphorylated tau (Wevers et al., 1999). $\alpha 7$ mRNA was upregulated in cholinergic neurons of the nucleus basalis in mild to moderate AD compared to patients with MCI or no impairments in cognition (Counts et al., 2007). Moreover, in AD patients who smoked tobacco, the $\alpha 4$ nAChR was upregulated in the temporal cortex compared to non-smokers with AD, but the $\alpha 4$, $\alpha 7$ and $\alpha 3$ nAChR levels

were all significantly lower than non-smoking controls (Mousavi et al., 2003). Interestingly, AD patients as well as controls who smoked have lower levels of brain A β 40 and A β 42 than controls (Hellstrom-Lindahl et al., 2004a).

In summary, both immunological detection methods and receptor binding studies have demonstrated deficits in nAChRs in AD that occur in multiple cortical and hippocampal brain regions without a consistent downregulation of nAChR mRNA. Importantly, these changes have been observed in mostly late stage disease and do not provide insight into the early mechanisms of disease pathogenesis.

There are inconsistent findings regarding the effect of AD on muscarinic cholinergic receptor (mAChR) expression. There is evidence of decreased mAChRs in AD, but this change is not consistent, is restricted to particular subtypes, and is less widespread compared to nAChRs. Table 1.2 summarizes the muscarinic cholinergic receptor changes present in AD. It is important to note that for studies of nAChR and mAChR changes in AD, several factors may contribute to differences found in brain regions analyzed, detection methods employed, and between the various published studies. Differences in patients from which brain tissues were obtained such as the demographics, the presence of other disease states, and the severity of disease may be a factor. Additionally, postmortem interval for tissue collection and techniques used may also account for some differences.

As a result of decades of research suggesting a link between cognitive impairment and cholinergic dysfunction, enhancement of the cholinergic system to combat the cognitive deficits of AD is employed through the use of (acetyl)cholinesterase inhibitors. The acetylcholinesterase inhibitors (AChEIs) were the first Food and Drug Administration (FDA) approved medications for the treatment of AD. Donepezil, rivastigmine, and galantamine, are examples that are currently in clinical use providing a modest symptomatic benefit (Nordberg and Svensson, 1998; Grutzendler and Morris, 2001; Cummings, 2004; Wilkinson et al., 2004), while the first approved agent, tacrine, is less often used due to its risk of hepatotoxicity (Watkins et al., 1994). By contrast, memantine, the newest drug approved for moderate to severe AD, acts as an antagonist at the N-methyl-D-aspartate (NMDA) receptor to target excitotoxic mechanisms theorized in the pathogenesis of neurodegenerative disease (Cummings, 2004; Parsons et al., 2007).

There is preclinical evidence that memantine also acts as an antagonist at $\alpha 7$ nAChRs (Aracava et al., 2005). Excitotoxicity is further discussed in the traumatic brain injury section of this chapter. Additionally, psychotropic medications including antidepressants and atypical antipsychotics are sometimes used to help treat AD related symptomatology (Cummings, 2004).

Cholinergic markers in early AD

Studies of the cholinergic system in subjects prior to the development of cognitive dysfunction and in patients with early AD are required in order to elucidate the series of pathogenic events causing dementia in AD and identify targets for therapeutic intervention. Mild cognitive impairment in humans occurs as a transitional period between normal aging and early AD in which patients exhibit memory impairment without dementia, disruptions in general cognitive function, or interference with the activities of daily living (Petersen et al., 2001). Interestingly, studies have shown preservation (Gilmor et al., 1999) or even upregulation of presynaptic markers such as ChAT in the hippocampus and frontal cortex in mild cognitive impairment (DeKosky et al., 2002; Ikonovic et al., 2003). However, researchers found a loss of immunoreactivity to the nerve growth factor receptor located on cholinergic basal forebrain neurons known as P75^{NTR}, which is vital for cholinergic neuron survival, in patients with MCI similar to that of patients with early AD, and this correlates with performance on cognitive scales (Mufson et al., 2002). A clinical trial recently conducted detected no loss of nAChRs through PET scans of patients with early AD using a radiotracer containing A-85380 selective for $\beta 2$ -containing subunits (Ellis et al., 2008). Nevertheless, another group found this radiotracer, which measures predominately $\alpha 4\beta 2$ receptors, to be reduced in both MCI and AD patients (Sabri et al., 2008). Interestingly, $\alpha 7$ nAChRs, as measured by [³H]-methyllycaconitine (MLA), demonstrated a non-significant elevation in patients with mild to moderate AD compared to patients with MCI or patients without cognitive impairment. Patients with whom a diagnosis of AD was confirmed by neuropathological analysis demonstrated elevated MLA binding and A β levels (Ikonovic et al., 2009).

Cholinergic impairments in transgenic mouse models of Alzheimer's disease

Extensive research has been conducted using transgenic mouse models aimed at elucidating mechanisms involved in AD related neuropathology. In addition, this allows researchers to better study early markers of disease instead of relying on tissue indicative of end-stage disease in order to better target therapeutic strategies. Several studies have demonstrated alterations in cholinergic function in transgenic animal models that express human genes containing APP mutations such as APP^{swe} alone or in combination with PS1 mutations. Many of the transgenic models engineered contain the Swedish double mutation on chromosome 21 in which an asparagine at amino acid 670 is substituted for a lysine, and at position 671, a leucine is substituted for a methionine. This mutation was named for a Swedish family in which many suffered from FAD (Mullan et al., 1992). Such models display elevated levels of A β expression including increased concentrations of soluble and insoluble A β and subsequent plaque deposition. Studies assessing cholinergic neuronal and synaptic markers have shown that prior to amyloid deposition, eight month old Tg2576 mice display a significant increase in the density of cortical cholinergic synapses as measured by vesicular acetylcholine transporter (VACHT) immunoreactivity (Wong et al., 1999; Hu et al., 2003) compared to wild-type mice. Tg2576, one of the most widely used AD mouse models, has a six fold higher expression of human APP than endogenous mouse APP. This results in a 14 fold increase in A β _{42/43}, a five fold increase in A β ₄₀, and subsequent amyloid deposition around nine to ten months of age (Hsiao et al., 1996; Pedersen et al., 1999). Eight month old mice expressing both APP^{swe} and PS1 (M146L) transgenes had severe amyloidosis, loss of cholinergic synapses in the neocortex and hippocampus (Wong et al., 1999) and decreased cortical VACHT expression (Hu et al., 2003). Twelve month old Tg2576 mice demonstrated enhanced p75 nerve growth factor receptor immunoreactivity in medial septal neurons indicating higher numbers of cholinergic basal forebrain neurons compared to non-transgenic mice as well as mice with both the APP^{swe} and PS1 transgenes (Jaffar et al., 2001). Cortical sections of 19 month old Tg2576 mice showed a degeneration of ChAT immunoreactive fibers located near amyloid plaques and activated glia (Luth et al., 2003). In addition, the presence of amyloid was associated with small decreases in AChE concentration *in vivo* as measured by microdialysis in 15 to 27 month

old mice expressing two different APP mutations including the Swedish mutation as well as a PS1 mutation (Hartmann et al., 2004). A study of the APP23 transgenic mouse reported a loss of neocortical ChAT and cholinergic fibers, a non-significant reduction in AChE, but no loss in cholinergic basal forebrain neurons in 24 month old transgenic mice (Boncristiano et al., 2002). There was also a decrease in cholinergic fiber length with aging in the mutant mice. Twelve month old APP23 mice also displayed losses in cholinergic fibers and AChE (Sturchler-Pierrat et al., 1997). These mice express the Swedish double mutation leading to a seven fold increase in APP production and the presence of amyloid deposition at six to eight months of age (Sturchler-Pierrat et al., 1997; Boncristiano et al., 2002; Boncristiano et al., 2005).

A number of studies have investigated changes in cholinergic receptor expression in mouse models of AD. Tg2576 mice demonstrated a reduction in cortical ^3H -cytisine binding representative of $\alpha 4\beta 2$ nAChR expression in 19 month old mutants (Apelt et al., 2002). Mice expressing APP^{swe}, PS1 and a tau mutation (3xTg-AD), displayed decreased BTX binding in the hippocampus, retrosplenial cortex, parietal cortex, and thalamus of six month old transgenic compared to non-transgenic mice. By six months of age, these transgenic mice display deficits in long term potentiation and synaptic dysfunction before plaque formation in the hippocampus (Oddo et al., 2003). Binding deficits in these regions were present along with intraneuronal accumulation of A β 42. Interestingly, in the auditory cortex, where no intraneuronal A β was detected, there was no downregulation in BTX binding. Conversely, no changes were found in non- $\alpha 7$ nAChRs, as measured by EPI binding, except for an upregulation in the thalamus of transgenic mice (Oddo et al., 2005). Another report showed a reduction in M1 mAChR binding as well as diminished HACU binding in five month old Tg2576 mice prior to the presence of amyloid deposition, but aged mice showed elevated cortical VAChT binding along with the deficits in choline uptake (Klingner et al., 2003). Cortical membrane preparations from three month old Tg2576 mice have shown downregulation of $\alpha 4$ nAChR mRNA, but no change in the mRNA of $\alpha 7$ or $\alpha 3$ receptor subunits (Mousavi et al., 2004).

In contrast, no change was found in $\alpha 7$ or $\alpha 4\beta 2$ nAChRs as measured by BTX and cytisine binding, respectively, in the parietal cortex and hippocampus of three week

to 17 month old APP^{swe} and PS1 double transgenic mice (Marutle et al., 2002). Furthermore, one group found no differences in cholinergic markers, such as ChAT and AChE activities and HACU and VAChT binding assays, in Tg2576 mice compared to littermates at 14, 18 and 23 months of age (Gau et al., 2002).

Surprisingly, further studies have reported an elevation in the expression of the $\alpha 7$ nAChR in Tg2576 mice. An upregulation in $\alpha 7$ nAChR protein expression in the hippocampal field CA1 and dentate gyrus with age, beginning at four months in the dentate, was found in Tg2576 mice compared to wild-type mice, but no such upregulation in $\alpha 4$ nAChR expression was apparent (Dineley et al., 2001). This group also reported an increase in hippocampal $\alpha 7$ nAChR protein expression in five month old Tg2576 mice, prior to A β deposition, as well as in an AD mouse model that contained the APP plus PS1 (A246E) gene mutations resulting in accelerated amyloid deposition around six to seven months of age (Dineley et al., 2002b). This finding was accompanied by deficits in the contextual fear learning behavioral task which examines hippocampal impairment. Furthermore, an increase in $\alpha 7$ nAChRs as measured by BTX binding was demonstrated in the cerebral cortex, hippocampus, caudate, and cerebellum of four month old Tg2576 mice preceding A β plaque formation and behavioral changes (Bednar et al., 2002). A similar upregulation was found in $\alpha 4\beta 2$ nAChRs as measured by cytosine binding but only in older mice, and no change was found in mAChR binding. Enhanced nicotinic receptor binding occurred along with an upregulation in the mRNA levels of both $\alpha 7$ and $\alpha 4$ receptor subunits. Tg2576 mice aged 14.5 months displayed increased BTX binding in cortex compared to wild-type mice (Hellstrom-Lindahl et al., 2004b). A group of transgenic mice treated chronically with nicotine for 5.5 months did not display this elevation in BTX binding (Hellstrom-Lindahl et al., 2004b) but, interestingly, showed a reduction in A β 42 immunopositive plaques by 80 percent (Nordberg et al., 2002; Hellstrom-Lindahl et al., 2004b). Further, with chronic treatment (Nordberg et al., 2002) and a shorter ten day treatment administered by the subcutaneous route (Hellstrom-Lindahl et al., 2004b), a decrease in insoluble A β 40 and A β 42 levels were noted. Conversely, in one month old mice expressing APP^{swe}, PS1 and a tau mutation (3xTg-AD), five months of nicotine exposure in the drinking water caused an

increase in EPI binding in both transgenic and wild-type mice but no change in BTX (Oddo et al., 2005). Nicotine did not affect amyloid, but appears to have exacerbated hyperphosphorylated tau related pathology. Finally, electrophysiologic studies performed on hippocampal slices from seven month old mice that express both APP^{swe} and PS1 mutations (M146V called TASTPM), in which A β deposition is accelerated and occurs at three months of age (Howlett et al., 2004), showed this model maintains functional $\alpha 7$ nAChRs located on GABAergic interneurons (Spencer et al., 2005).

To summarize, initial studies in several transgenic mouse models of AD mostly showed deficits in nAChRs and other cholinergic indices; however, presynaptic markers were found to be upregulated prior to the presence of amyloid deposition. Further studies revealed upregulation or a sparing of nAChR in various models depending on brain region, nAChR subtype, and age; in particular, this occurs in younger mice prior to the development of A β neuropathology. Therefore, inconsistencies in the alterations of nAChR density in AD related models may be due to differences in the transgenic models, the techniques employed for the assessment of AChRs, the brain regions evaluated, or the age of the animals relative to the development of pathology.

The relationship between cholinergic neurotransmission and A β

The A β -nAChR interaction. Recently, a direct pharmacological interaction between A β and the $\alpha 7$ nAChR has been established. In the brains of patients with AD, immunoprecipitation studies have shown that A $\beta 42$ and the $\alpha 7$ nAChR, but not the $\alpha 4$ or other subunits, are co-localized in neuritic plaques as well as in cortical and hippocampal neurons in which they interact with high affinity and form stable complexes (Wang et al., 2000b). This interaction has also been demonstrated in human and rodent cell culture models. Further experiments in human neuroblastoma, SK-N-MC, cells transfected with the $\alpha 7$ nAChR showed that BTX and methyllycaconitine (MLA), both selective $\alpha 7$ nAChR antagonists, inhibited the binding of A $\beta 40$ with an IC₅₀ of 1pM and 4pM, respectively, for the higher affinity of two binding sites, and A $\beta 42$ bound with an affinity in the low femtomolar range (IC₅₀ of 8fM). A β binding resulted in cytotoxicity which was attenuated by nicotinic agonists suggesting this ligand-receptor interaction may be important in the pathogenesis of AD. This interaction was further confirmed in rat

synaptic membranes in which selectivity for the $\alpha 7$ receptor subtype was demonstrated (A β 42 inhibition of MLA and cytosine binding, respectively: K_i of 4.1 pM for $\alpha 7$ and K_i of 30 nM for $\alpha 4\beta 2$) (Wang et al., 2000a).

Controversy exists as to whether A β is acting as an agonist or antagonist at the $\alpha 7$ nAChR; however, A β may elicit varying responses depending on factors such as A β concentrations and the level of disease progression (Dineley et al., 2002a; Hernandez et al., 2010; Jurgensen and Ferreira, 2010). Several studies have demonstrated A β 's agonist-like behavior. In hippocampal slices prepared from mice containing the desensitization resistant mutant (L250T) $\alpha 7$ nAChR, nicotine produced activation of the mitogen-activated protein kinase (MAPK) cascade, involved in learning and memory, followed by downregulation or desensitization (Dineley et al., 2001). Stimulation of this pathway was blocked by the selective $\alpha 7$ antagonist, MLA. In cultured rat hippocampal neurons, A β 42 at physiologic concentrations activated the MAPK pathway and prevented the stimulatory effects of nicotine, suggesting that A β and nicotine may act at a common receptor. The selective $\alpha 7$ antagonists, MLA and BTX, inhibited A β 's actions which were also dependent on Ca^{2+} suggesting the involvement of the $\alpha 7$ receptor and its necessary function since this receptor is highly permeable to Ca^{2+} . Electrophysiology studies showed that rat A β 42 in a soluble, non-aggregated form activates rat $\alpha 7$ nAChR expressed in *Xenopus* oocytes causing inward currents and Ca^{2+} influx while A β 40 produced a similar effect but to a lesser degree, and MLA blocked this effect (Dineley et al., 2002a). Addition of the L250T mutation showed that A β acts as an agonist at the $\alpha 7$ nAChR at low (pM) concentrations activating the receptor with subsequent desensitization to A β but not nicotine, and at higher concentrations (nM) A β produces inactivation with cross desensitization to nicotine. In rat hippocampal and neocortical isolated presynaptic terminals, A β 42 activated nAChRs causing increases in presynaptic Ca^{2+} (Dougherty et al., 2003). The $\alpha 7$ receptor was activated at picomolar concentrations and non- $\alpha 7$ receptors became activated at nM concentrations. A β inhibited nicotine activation of presynaptic nAChRs and A β 's effect was blocked by other antagonists suggesting A β binds competitively (Dougherty et al., 2003), whereas others have

suggested A β binds to two different binding sites (Wang et al., 2000b; Wang et al., 2000a; Dineley et al., 2002a) or binds non-competitively (Liu et al., 2001).

Additionally, A β binds to non- α 7 nAChRs, which in the mammalian brain are mainly comprised of the α 4 β 2 subtype, but with much lower affinity (Wang et al., 2000a). However, soluble A β 42 and A β 25-35 have been shown to act as ligands at non- α 7 nAChRs in rat basal forebrain neurons at low doses (fM to pM), and patch-clamp recordings show activation of this subtype on postsynaptic neurons of the diagonal band of Broca within the basal forebrain (Fu and Jhamandas, 2003).

In contrast, there is also evidence that A β acts as an antagonist at the nAChR. This is supported by studies showing an overall deficit in cholinergic neurotransmission as described above or through direct antagonist properties at receptors. Non-aggregated A β 42 at nanomolar concentrations was shown to reversibly block the function of the α 7 receptor without displacing BTX binding in rat hippocampal pyramidal neurons (Liu et al., 2001). Voltage clamp studies in *Xenopus* oocytes expressing human wild-type and mutated α 7, the L248T mutation which gives agonist activity to antagonists, showed A β 42 blocked ACh-evoked currents in wild-type α 7 nAChRs but activated the mutant receptors (Grassi et al., 2003). Most recently, patch clamp electrophysiology studies documented the partially reversible α 7 antagonist properties of A β 42 in a rat pituitary tumor cell line expressing either human or mouse α 7 receptors (Spencer et al., 2005). In rat frontal cortical synaptosomes, A β reduced K⁺-stimulated ACh release and nicotine induced calcium influx by 75 percent. A β 42 decreased the function of α 7 in an irreversible manner whereas A β 40 produced a milder, reversible inhibition (Lee and Wang, 2003). Nanomolar concentrations of A β 40 inhibited K⁺-evoked ACh release in hippocampal slices from young and aged rats, and aged rats demonstrating cognitive impairments, measured by Morris water maze performance, showed greater sensitivity to this effect (Vaucher et al., 2001). Intracerebroventricular injection of A β 42 resulted in antagonism of α 7 nAChRs, as the peptide blocked a hemodynamic response elicited by brain injection of the selective α 7 agonist, choline, confirming A β 's actions *in vivo* (Li and Buccafusco, 2004).

A β 42 at nanomolar concentrations blocked both postsynaptic α 7 and to a greater degree non- α 7 nAChR currents on hippocampal interneuron slice preparations (Pettit et al., 2001). A β 40 and 42 inhibit whole cell membrane currents of α 4 β 2 and to a lesser extent α 7 nAChRs expressed in *Xenopus* oocytes as well as in the *Torpedo* electric organ model of muscular nAChRs (Tozaki et al., 2002). Patch clamp studies in SH-EP1 cells heterologously expressing α 4 β 2 nAChRs revealed that A β 42 inhibits function with much higher affinity for the α 4 β 2 receptor than α 7 receptors in a non-competitive manner (Wu et al., 2004). In addition, in rat cortical neurons, A β impaired muscarinic receptor activity by the inhibition of G-protein coupling to M1, M3, and M5 mAChRs. A β caused impaired carbachol induced GTPase activity, inositol phosphate accumulation, and Ca²⁺ release from intracellular stores, all without toxicity to neurons (Kelly et al., 1996).

The contradictory properties of A β at nAChRs may be due to differences in the tissue models, experimental conditions such as A β concentrations, or detection methods employed. Notably, in a recent report a unique nAChR subtype, α 7 β 2, was discovered in rat basal forebrain cholinergic neurons, and A β demonstrated high affinity binding to this subtype and functional inhibition (Liu et al., 2009).

A β modulates cholinergic receptor expression and neurotransmission in vitro. *In vitro* studies have shown alterations in nAChR expression following A β exposure. Exposure of cultured rat hippocampal slices with A β 42 caused a two fold increase in α 7 nAChR expression (Dineley et al., 2001). By contrast, in PC12 cells that do not express the α 4 nAChR, both A β 40 and A β 25-35 treatment resulted in a decrease in α 3 and α 7 nAChR protein, EPI and BTX binding (Guan et al., 2001; Guan et al., 2003), and a decrease in α 3 and α 4 nAChR mRNA. Further, β 2 nAChR protein expression was reduced; however, only the A β 25-35 fragment resulted in a reduction in β 2 mRNA. (Guan et al., 2001). The disparity in nAChR expression between models may be due to the difference in cell type or the use of aged or aggregated A β peptide. The latter group used this method by which A β was heated to 37°C for a period of time forming soluble oligomers. In rat primary hippocampal cells, aged A β 42 caused no change in BTX binding in areas where fibrils were detected, although this resulted in toxicity including retraction of dendrites, cell body shrinkage, but no change in neuronal number (Lain et

al., 2005). In primary rat cortical and hippocampal astrocytes, aged A β 42 exposure for 48 hours caused an upregulation in α 7, α 4, and β 2 mRNA and protein expression (Xiu et al., 2005).

The A β peptide is implicated in the dysfunction of cholinergic neurotransmission. In cortical and hippocampal rat brain slices, non-aggregated A β peptides inhibited K⁺ - mediated ACh release (Kar et al., 1996; Kar et al., 1998), and in synaptosomes from the hippocampus, frontal cortex, and striatum, decreased high affinity choline uptake (HACU) without altering ChAT (Kar et al., 1998). In addition, A β 42 inhibits K⁺-evoked ACh release from rat cortical synaptosomes (Wang et al., 1999a) and electrophysiological studies in acutely dissociated rat cholinergic basal forebrain neurons from the diagonal band of broca, showed that A β (40 and 25-35) decreased K⁺ channel currents and increased depolarization. RT-PCR showed this effect to be selective to cholinergic neurons (Jhamandas et al., 2001). In immortal rat cell lines expressing a cholinergic phenotype, A β resulted in a dose dependent inhibition of mitochondrial function while cells expressing a serotonergic phenotype were not affected by this toxicity (Olesen et al., 1998). In primary rat septal neurons, aggregated A β 40 and 25-35 caused neurotoxicity and up to a 60 percent decrease in ChAT activity (Zheng et al., 2002). In addition, aggregated A β 42, but not fresh A β 40 or A β 42, has been shown to increase AChE activity by up to 35 percent in mouse primary cortical neurons without eliciting toxicity. This effect was enhanced by α 7 nAChR agonists and blocked by antagonists (Fodero et al., 2004). In the SN56 mouse cell line model of basal forebrain cholinergic neurons, non-aged A β peptides under non-toxic conditions caused reduced ACh content, ChAT activity, but no change in AChE activity (Pedersen et al., 1996). This finding was confirmed in primary rat septal and basal forebrain cholinergic neurons in which non-aged A β 42, but not A β 40 at physiologic concentrations of 10nM suppressed intracellular ACh levels and ACh synthesis rate, and 100nM caused a decrease in pyruvate dehydrogenase (PDH) activity, an enzyme that converts pyruvate to acetyl-CoA needed for ACh synthesis. In these cells, A β 42 was not cytotoxic and caused no change in choline uptake, AChE activity or staining, or ChAT activity (Hoshi et al., 1997).

Exogenous A β disrupts cholinergic function and cognitive behavior in rodents.

An alternative to the use of transgenic mice for the study of Alzheimer pathophysiology involves direct A β administration into the brains of rodents. Exogenous delivery of A β through intracerebroventricular (i.c.v.) administration, either by single injection or osmotic mini-pump infusion impairs cholinergic neurotransmission and behavioral indices of memory and cognition. Intracerebroventricular administration of the A β peptide in rats resulted in a decrease in cortical and hippocampal ChAT activity (Nabeshima and Nitta, 1994; Nitta et al., 1994), a decrease in nicotine-evoked nAChR depolarization and release of ACh in the frontal cortex and hippocampus (Itoh et al., 1996; Olariu et al., 2001; Tran et al., 2001; Tran et al., 2003), but no change in AChE (Nabeshima and Nitta, 1994). Impairments in cognitive assessments including Morris water maze (Nabeshima and Nitta, 1994; Nitta et al., 1994; Olariu et al., 2001), passive avoidance (Nabeshima and Nitta, 1994; Olariu et al., 2001), and radial eight arm maze tasks (Tran et al., 2001) were observed in addition to an increase in anxiety (Olariu et al., 2001). However, injection of A β directly into the cortical and hippocampal parenchyma revealed toxicity confined to the site of injection which was not selective to cholinergic neurons (Emre et al., 1992). When injected into the medial septum of rats, A β caused a decrease in basal and K⁺-evoked ACh release and a decrease in septal ChAT immunoreactive neurons (Harkany et al., 1995) with no change in septal or cortical ChAT activity (Abe et al., 1994). Aged A β injected into the nucleus basalis resulted in a fibril deposit along with decreases in basal and K⁺-stimulated ACh release in the parietal cortex, ChAT immunoreactivity, and performance in the object recognition task of cognition (Giovannelli et al., 1995). In mice, i.c.v. injection of the APP cleavage product, the carboxy-terminal fragment (CT105), which is the peptide produced from the actions of β -secretase before γ -secretase acts to release the A β peptide, resulted in a decrease in hippocampal and cortical ACh levels. PDH activity was reduced after exposure to either CT105 or the A β peptide following i.c.v injection, but there was no change in ChAT or AChE activities. In addition, spatial memory performance in the Morris water maze and the Y-maze task was impaired more severely in the CT105 treated mice compared to A β treated mice (Choi et al., 2001).

Cholinergic neurotransmission regulates APP processing. Neuronal activity, in particular cholinergic neurotransmission, has been implicated in the regulation of APP processing. So not only does the interplay between the cholinergic system and amyloid pathology in AD involve A β 's effect on cholinergic neurotransmission, the loss of cholinergic innervation leads to aberrant APP processing which may exacerbate pathology. This is demonstrated in animal models of cholinergic deafferentation. Selective immunolesions of the rat basal forebrain with unilateral cerebroventricular injection of 192 IgG-saporin conjugated to the low affinity growth factor receptor specific to cholinergic neurons caused a bilateral loss (90-95 percent) of ChAT positive cholinergic neurons of the basal forebrain (Leanza, 1998; Lin et al., 1999), and cholinergic denervation as measured by AChE staining (Leanza, 1998; Lin et al., 1998; Lin et al., 1999). A decrease in AChE staining occurred in the nbM, septal nucleus (horizontal limb of the diagonal band) (Lin et al., 1998), frontal cortex, and hippocampus (Lin et al., 1998; Lin et al., 1999). This lesion also caused APP expression increases of 20 percent in the frontal cortex and 30 percent in the CA3 subfield of the hippocampus (Lin et al., 1998), and one study showed an increase in expression of up to 70 percent in the cortex and hippocampus (Leanza, 1998). In this model, downregulation of AChE expression in the frontal cortex and CA3 correlated with enhanced APP expression and greater impairments in Morris water maze performance (Lin et al., 1998). In rabbits, this method led to A β deposition and increases in cortical A β 40 and A β 42 (Beach et al., 2000). Excitotoxic lesions of the rat nbM with *N*-methyl-D-aspartic acid, resulting in decreased ChAT and AChE activities, caused an increase in cortical APP measured in *ex vivo* polysomal systems (Wallace et al., 1991) due to an increase in mRNA synthesis (Wallace et al., 1993). But, this laboratory found that serotonergic and noradrenergic lesions also caused enhanced APP expression (Wallace et al., 1993; Wallace et al., 1995). In addition, cholinergic and serotonergic lesions caused enhanced secretion of APP in CSF, and this effect was most pronounced in aged nbM lesioned rats (Wallace et al., 1995). The elevated CSF levels of secreted APP in cholinergic lesioned rats was a soluble form of APP that did not contain the cytoplasmic carboxy-terminal fragment, but a significant portion contained amino acid residues 1-28 of the A β peptide, while low A β peptide was detected. There was also an increase in the carboxy-terminal fragment in the

cerebral cortex (Wallace et al., 1995). Fimbria-fornix transection, a functional hippocampal lesion, resulted in accumulation of APP immunoreactivity near cholinergic fiber degeneration in the hippocampus of rats (Beeson et al., 1994); however, in mice overexpressing both human APP and PS1, the lesion did not alter the processing or expression of APP when tested out to 18 months of age (Liu et al., 2002b; Liu et al., 2002a). Nevertheless, Morris water maze impairments were noted in lesioned and transgenic mice (Liu et al., 2002b). In contrast, the application of current flow to produce cholinergic denervation in mice overexpressing human APP (the APP23 model) resulted in reduced A β levels in the lesioned hemisphere (Boncristiano et al., 2002).

Combining the MCI findings, the data indicating an early elevation in nAChR expression in AD mouse models, and the data demonstrating the interaction of α 7 nAChRs and A β suggest the α 7 nAChR protein may be an early target in Alzheimer's disease. Our laboratory was interested in determining if nAChR changes occur as an early event prior to the development of neuropathology or if receptor alterations occur as a late consequence of disease. This leads to the first research question addressed by the dissertation: Are changes in the α 7 nicotinic acetylcholine receptor an early marker or a late consequence in experimental Alzheimer's disease? Transgenic mouse models may be used for mechanistic studies to investigate the cause and effect relationship between dementia and nAChR loss. Loss of nAChRs early in the disease process may be the cause of dementia or dementia and the associated neuropathological processes may be the cause of the loss of nAChR expression. An early loss of nAChRs would suggest a possibility for therapeutic benefit through pharmacological interventions targeting nAChRs.

Traumatic Brain Injury

Alzheimer's disease and traumatic brain injury are linked

The study of Alzheimer's disease and traumatic brain injury (TBI) is invariably linked through a similarity in pathophysiological mechanisms, a shared disruption in cognitive function, and a potential causal relationship. Alzheimer's disease and traumatic brain injury share common pathophysiological processes that contribute to neuronal dysfunction and cell death. For example the immune response following TBI and in the

presence of AD is a significant focus of research. Inflammatory mediators including proinflammatory cytokines, prostaglandins, free radicals, complement, and the subsequent activation of microglia, are involved in disease progression (Sheng et al., 1998; Aisen et al., 2003; Lucas et al., 2006). Mitochondrial dysfunction and further oxidative stress also occurs (Querfurth and LaFerla, 2010). There has been an extensive investigation of the role of excitotoxicity in which most of the attention has focused on enhanced glutamate neurotransmission. Excitotoxic mechanisms involve intracellular calcium ion accumulation resulting in oxidative damage of lipids and proteins (Mattson, 2003; Arundine and Tymianski, 2004; Mattson, 2007; Forder and Tymianski, 2009). Research in experimental models has been successful and invaluable in contributing to an understanding of the mechanisms involved. However, most of these therapeutic modalities have not been proven to be beneficial in the clinical setting with the exception of memantine targeting excitotoxicity for the treatment of AD.

Similarly to the symptomatology of AD, individuals living with TBI experience alterations in learning, memory, and behavior. Because of its involvement in synaptic plasticity, deficits in the cholinergic system, have been shown to mediate decrements in cognitive processes (Levin and Simon, 1998; Paterson and Nordberg, 2000). A loss of glutamate activity, a key mediator of synaptic plasticity, also contributes to cognitive impairments (Schaeffer and Gattaz, 2008). Reductions in glutamate neurotransmission in early Alzheimer's disease are thought to cause deficits in synaptic transmission. The seemingly contradictory mechanisms of neurotransmitter deficits and excitotoxic processes underscore the complexity of these disease states.

Many studies have been done to ascertain the association between a history of TBI and the development of AD. Evidence has been derived from histopathological studies utilizing tissues from head injury cases, retrospective analyses, and cohort studies; extensive reviews of these data can be found in the following references (Lye and Shores, 2000; Van Den Heuvel et al., 2007). The first case report was documented in 1982 in which a 38 year old male presented with early onset AD 16 years after experiencing a severe TBI (Rudelli et al., 1982). A retrospective analysis showing a positive link between head injury and dementia of the Alzheimer type followed soon thereafter

(French et al., 1985). Since then, numerous epidemiological studies have confirmed this association while other studies could not establish a correlation.

Pathologic changes reminiscent of AD have been observed following TBI. Some studies have demonstrated the presence of A β deposition, while in other studies A β deposition has been limited and was not present in younger individuals (Van Den Heuvel et al., 2007). TBI is associated with APP accumulation in damaged axons (Gentleman et al., 1993), and the colocalization of A β within axons has also been observed (Smith et al., 2003) and may lead to the formation of extracellular plaques. In patients with severe TBI and the presence of diffuse axonal injury, A β 42 and APP was significantly elevated in the CSF, but not in the plasma, within days of the injury (Olsson et al., 2004). In patients sustaining a severe TBI, surgical resection of the lesioned temporal cortex demonstrated diffuse A β deposits in a third of the cases within hours following injury (Ikonovic et al., 2004). In a similar design, patients who demonstrated the presence of diffuse A β plaques (A β immunoreactivity in the extracellular space) in excised temporal lobe displayed greater concentrations of A β 42, but not A β 40, compared to patient tissues without plaques. In addition, tissues with plaques showed a higher A β 42 to A β 40 ratio, and one half displayed the apolipoprotein E4 (APOE ϵ 4) allele, a susceptibility factor for developing AD (DeKosky et al., 2007). By contrast, one study found an absence of A β plaques but instead long term axonal deposition of A β in autopsied brains of patients who survived up to 3 years past injury. The authors postulated that A β deposition immediately after injury may be removed through catabolic processes involving an A β degradation enzyme called neprilysin and that this process may be hindered by aging (Chen et al., 2009).

The APOE ϵ 4 allele is associated with A β deposition following TBI and has been associated with a poor outcome following TBI (Teasdale et al., 1997; Van Den Heuvel et al., 2007). In addition, patients possessing the APOE ϵ 4 allele had a ten fold greater risk of being diagnosed with AD after sustaining a TBI (Mayeux et al., 1995; Van Den Heuvel et al., 2007). This evidence is not conclusive, for other studies have not shown an influence on outcome following TBI or an association with the development of AD (Jellinger, 2004; Mauri et al., 2006; Van Den Heuvel et al., 2007). Lastly, *in vivo* models

have been employed to investigate the effects of TBI on the development of AD-like pathology and behavior. For example, repetitive mild brain trauma (two controlled cortical injuries 24 hours apart) hastens A β accumulation and increases A β 42 and A β 40 concentrations in nine month old Tg2576 mice compared to a single injury (Uryu et al., 2002).

Recently sports related TBI, namely concussion, among players in the national football league (NFL) has gained awareness. An NFL sponsored telephone study of 1063 retired NFL players showed that players under 50 years of age had a 19 fold increase in AD or other memory impairments after experiencing concussion (Weir et al., 2009). In a previous study of retired NFL players, multiple concussions were directly related to the diagnosis of mild cognitive impairment and self-reported memory impairment, and retired players had an earlier onset of AD (Guskiewicz et al., 2005). Athletes who have experienced concussion and subconcussive injuries and display behavioral and cognitive symptoms later in life have what is known as chronic traumatic encephalopathy (CTE). CTE was originally termed dementia pugilistica and was first demonstrated in boxers who developed memory impairments, parkinsonism, and behavioral and mood alterations. CTE has a slow progression, as the brain displays atrophy and neurofibrillary tangles staining positive for tau protein. However, diffuse A β plaques are much less common (McKee et al., 2009). Considering the recent findings in the study of sports related TBI, collectively, the literature is supportive of TBI predisposing one to the development of dementia like syndromes or AD.

Traumatic brain injury background

Each year in the U.S. about 1.4 million individuals sustain a traumatic brain injury (TBI) leading to 50,000 deaths and 235,000 hospitalizations (Langlois et al., 2006). Traumatic brain injury frequently occurs in males ages 15-24, children ages five and younger, and senior citizens ages 75 and older (NINDS, 2002). TBI is most commonly caused by motor vehicle accidents, as well as pedestrian and bicycle accidents, violence, and falls (in the elderly) (NINDS, 2002; Langlois et al., 2006). Traumatic brain injury is classified as mild, moderate, or severe using the Glasgow Coma Scale (Teasdale and Jennett, 1974) that evaluates coma and impairments in consciousness by assessing eye

opening, motor response, and verbal response. A score of 13-15 classifies a patient as having a mild form of injury. A score of 9-12 indicates a moderate injury. A score of 3-8 and below classifies a patient with severe TBI. (Saatman et al., 2008) (NINDS, 2002).

Mild traumatic brain injury is the most common form of head injury occurring in about 75 percent of cases and is also known as concussion (NCIPC, 2003). Concussion occurs when an impact to or forceful motion of the head causes a brief alteration in mental status or loss of consciousness. Mild TBI may also present as headache, dizziness, confusion, blurred vision, tinnitus, changes in mood and behavior, and difficulties with memory, attention and thinking. In the past few years, TBI has received an increase in awareness due to its prevalence in combat in the wars in Iraq and Afghanistan, as TBI has been referred to as the “signature injury” of the war (Hoge et al., 2008). Combat related mild traumatic brain injuries, many of which are characterized as blast injuries, are associated with post-traumatic stress disorder, depression, and other health concerns. In addition, mild TBI is receiving more attention as athletes and school children participating in sports are afflicted.

Moderate to severe injuries can include skull fractures, which may cause contusions or bruising of the brain parenchyma (NINDS, 2002). A penetrating injury occurs when a foreign object breaks through the skull and causes damage to brain tissue including cavitation of the tissue if traveling at a high velocity (Chesnut, 2007; Ling and Marshall, 2008). This type of injury occurs in only 10 percent of cases, almost all due to firearms, but is 90 percent fatal (Thurman et al., 1999). Conversely, a depressed skull fracture may result in contusion without a penetrating wound (Chesnut, 2007). Most injuries are referred to as non-penetrating or closed injuries. For example, the coup and contrecoup injuries are caused by movement of the brain within the skull resulting in contusions at the point of impact and distal to the impact area, respectively (NINDS, 2002; Gaetz, 2004). Additionally, diffuse axonal injury occurs from a rotation or stretching (shearing in the most severe cases) of the white matter, and a less diffuse, limited form is known as traumatic axonal injury (LaPlaca et al., 2007; Saatman et al., 2008). Furthermore, hematomas may present as bleeds within the brain parenchyma called intracerebral hematomas or within the meninges, known as epidural and subdural hematomas (NINDS, 2002). In addition to clinical severity of injury and causal factors

for the injury, traumatic brain injury can be classified by four pathoanatomic outcomes based on imaging and autopsy findings. These include contusions, subarachnoid hemorrhage, hematomas and diffuse axonal injury (Saatman et al., 2008). Glasgow coma scale score, age, pupillary diameter and light reflex, hypotension, and pathoanatomy are early predictors of outcome in severe TBI (Marion, 2006).

TBIs are also defined as either focal or diffuse in order to describe mechanisms involved in pathophysiology and investigate potential interventions. A focal injury is due to an impact (loading) force and includes skull fractures, epidural hematomas, and brain contusions. A diffuse injury is due to an inertial force and includes concussion, subdural hematoma, and diffuse axonal injury (LaPlaca et al., 2007; Saatman et al., 2008). *In vivo* models of experimental TBI include both focal and diffuse methodologies (LaPlaca et al., 2007). Two widely used methods of focal injury resulting in a contusion injury are the controlled cortical impact (Lighthall, 1988; Dixon et al., 1991; Goodman et al., 1994) and the fluid percussion injury (Dixon et al., 1987), which displays elements of a diffuse injury as well. Other paradigms include the weight drop model of focal injury and the impact acceleration (Marmarou et al., 1994) and rotational acceleration models (Smith et al., 2000) of diffuse injury. Common animal models employed in experimental TBI are reviewed by Cernak (2005).

Traumatic brain injury involves two pathophysiological processes. The primary injury occurs at the moment of impact with mechanical damage to cell membranes affecting neuronal cell bodies, axons and blood vessels (Bramlett and Dietrich, 2007). In just hours following injury, the cerebral cortex, hippocampus, thalamus and substantia nigra are susceptible to neural impairment and cell death (McIntosh et al., 1998). Further, a secondary injury follows involving a number of pathophysiological processes varying in onset and duration, some acute and some chronic, lasting up to one year. This process occurs in more severe injuries, as a mild TBI would be less likely to generate the full scope of secondary injury mechanisms. This secondary injury includes ischemia, calcium mediated excitotoxicity, mitochondrial dysfunction, free radical production, altered cerebral blood flow, edema, intracranial hypertension, cell death, and inflammation (Bramlett and Dietrich, 2007; Greve and Zink, 2009). Early disruptions in the blood brain barrier and decreased cerebral blood flow occur. Cerebral blood flow

falls due to reductions in cerebral perfusion pressure which leads to ischemia and altered metabolism as well as vasogenic and cytotoxic edema resulting in neuronal death due to necrosis. The presence of edema and hematoma causes elevations in intracranial pressure and further decreased cerebral blood flow.

Excitotoxicity has been the focus of extensive study (Mattson, 2003). The most widely studied mediator has been glutamate and its activation of NMDA receptors (Arundine and Tymianski, 2004). Other receptor systems highly permeable to calcium such as the $\alpha 7$ nAChR may be involved, as an increase in cholinergic activity measured by elevated ACh has been documented immediately following experimental injury (Saija et al., 1988). However, Biegon et al. (2004) found a 50 percent loss in glutamate NMDA receptors in a mouse model of closed head injury by one hour following injury, and agonist stimulation of the NMDA receptor 24 and 48 hours following injury provided cognitive improvement out to 14 days of testing. Thus, the authors concluded there is a short window of hyperexcitability for treatment with antagonist. The excitotoxic process is initiated by a massive neuronal depolarization and release of excitatory amino acid neurotransmitters following TBI (Greve and Zink, 2009). Depolarization of neurons, glia, and vascular endothelial cells causes excessive glutamate release and NMDA receptor stimulation associated with calcium influx. Overactivity of intracellular calcium ion causes protein phosphorylation and mitochondrial dysfunction (Sullivan et al., 2005) thereby increasing reactive oxygen species, lipid degradation, proteolysis, ATP dysfunction, and apoptosis (McIntosh et al., 1998; Greve and Zink, 2009). In addition, NMDA receptor activation causes nitric oxide production and further generation of peroxynitrite through reaction with mitochondrial superoxide anions contributing to DNA fragmentation, lipid peroxidation and amino acid breakdown (Greve and Zink, 2009). Additional oxidative damage occurs through hydrogen peroxide formed by catecholamine degradation and calcium induced arachidonic acid formation and subsequent free radical production. Excess iron concentrations due to hemorrhage are available for catalysis of free radical products (Greve and Zink, 2009). Further, the calcium dependent proteases, calpains, induce cytoskeletal degradation and impaired axonal transport.

Chronic inflammation involves upregulation of the proinflammatory cytokines, interleukin-1, interleukin-6, and tumor necrosis factor. This in turn, results in receptor expression inducing microglial activation and inflammatory cell signaling cascades which are associated with programmed cell death (Bramlett and Dietrich, 2007). While preclinical investigations have been successful, NMDA receptor antagonists targeting glutamate excitotoxicity and steroids aimed at reducing inflammation have not demonstrated clinical benefit (Hatton, 2001; Narayan et al., 2002).

Treatments targeting the mediators of secondary injury have been studied, but to date, no drugs are clinically approved for neuroprotection or enhancement of functional recovery in TBI patients. The mainstay of treatment continues to be structured clinical management of the patient with supportive care such as cerebral hemodynamic care and prevention of infections, seizures, and deep vein thrombosis which all may occur in the hospitalized TBI patient (Hatton, 2001). Clinical guidelines for the treatment of severe TBI have been formulated using evidence based medicine (Marion, 2006; Chesnut, 2007; Ling and Marshall, 2008). Resuscitation, intubation, and cardiac life support are first employed. Computed tomography (CT) imaging is used to characterize the injury and direct treatment. Oxygenation can be achieved by use of supplemental oxygen or endotracheal intubation. Fluid infusion can be used to resuscitate blood pressure, and cerebral perfusion pressure (CPP) must be maintained to prevent tissue damage. CPP can be raised by elevating the mean arterial pressure or lowering the intracranial pressure. An intracranial pressure (ICP) monitor is inserted, ICP and CPP are monitored, and treatment of intracranial hypertension is initiated if needed. ICP is managed through the use of mannitol, CSF drainage, hyperventilation induced vasoconstriction, surgical resection of the lesion, or decompressive craniectomy. Nutritional support and routine ICU care are also employed. Seizure prophylaxis within seven days of the TBI with phenytoin or valproic acid is initiated (Bratton et al., 2007d). Prevention of deep vein thrombosis with compression stockings or use of low molecular weight heparin or unfractionated heparin (Bratton et al., 2007b) is also employed. The use of jugular venous saturation or brain tissue oxygen monitoring for the measurement of cerebral oxygenation (Bratton et al., 2007a) is now recommended. Lastly, the use of hypothermia may improve outcome (Bratton et al., 2007c).

Approximately 5.3 million Americans are living with long term functional changes in thinking, sensation, language, and emotion as a result of TBI (Thurman et al., 1999; NINDS, 2002). In 2005, it was estimated that approximately one percent of the US civilian population suffers from long term disabilities due to TBI (Zaloshnja et al., 2008). Characterized by deficits in arousal, attention, memory, and executive functioning, impairments in cognition are among the neurobehavioral/neuropsychiatric sequelae of TBI (Rao and Lyketsos, 2000; Arciniegas, 2003). Executive function is defined as the ability to initiate, prepare, and make goals, know the consequences of those goals, and adapt to the outcomes (NINDS, 2002; Warden et al., 2006; Tsaousides and Gordon, 2009). It involves organizing, problem solving, and abstract reasoning (NINDS, 2002; Warden et al., 2006; Tsaousides and Gordon, 2009).

Arciniegas and McAllister (2008) reviewed the neurobehavioral symptoms that can occur following TBI, known as posttraumatic encephalopathy, with a focus on more severe injuries. Injuries to the entorhinal cortex and hippocampus can result in deficits in attention, working (short term), and declarative (pertaining to facts) memory. When the amygdala is affected, emotional and social behavior abnormalities occur. In particular, damage to the ventral forebrain disrupts cholinergic inputs to the neocortex and medial temporal lobe resulting in cognitive dysfunction including difficulties with attention, memory, and executive function. When the reticular formation within the brain stem is involved, neurotransmission is disrupted including ACh tracts as well as dopamine, norepinephrine, serotonin, glutamate, and GABA (γ -aminobutyric acid) pathways between the cortex, thalamus, and spinal cord causing impaired consciousness, arousal, and attention. Injury to the thalamus results in dysfunction of sensory and motor processing. In the hypothalamus, lesions result in autonomic dysfunction of thermoregulation and feeding, endocrine impairments, and sleep-wake aberrations. In the frontal cortex, involvement of specific subregions may lead to disinhibition, irritability and aggression, impaired executive function and working memory, or apathy. White matter involvement causes a disruption in information processes regarding emotion, cognition, and behavior. Silver and coworkers (2009) reviewed these relationships similarly and noted that some individuals with mild TBI may be affected in the same manner.

Patients with TBI of all severity levels may experience neurobehavioral symptoms including cognitive disorders, altered personality, major depression, anxiety disorders including post traumatic stress disorder, substance abuse, headache, dizziness, nausea, fatigue, sleep disturbances, and seizures that may be addressed pharmacotherapeutically (Riggio and Wong, 2009). Depression and cognitive impairment are most common following mild TBI; depression can be treated with the SSRIs, citalopram and sertraline, and cognitive impairment has been treated with the AChEIs, rivastigmine and donepezil, and the psychostimulant, methylphenidate (Silver et al., 2009). Current therapeutic approaches are reviewed by the neurobehavioral guidelines working group (Warden et al., 2006). The use of AChEIs in TBI is discussed in greater detail later in this chapter.

Cholinergic abnormalities in human and experimental traumatic brain injury

Similar to AD, both clinical and experimental investigations have demonstrated a disruption in cholinergic neurotransmission following TBI. Functional neurologic recovery from experimental brain injury in rats inducing motor dysfunction, has been shown to involve the basal forebrain cholinergic system and cholinergic mediated cortical plasticity and motor learning (Conner et al., 2005). In a clinical study, imaging and neuropsychological examinations linked the cognitive sequelae of TBI to the basal forebrain (Salmond et al., 2005). Postmortem brain studies of individuals with fatal head injuries showed deficits in cortical and cingulate gyrus ChAT activity and cingulate synaptophysin immunoreactivity, a measure of synapses, both indicative of presynaptic cholinergic deficits following TBI (Murdoch et al., 1998). In addition, cortical cholinergic innervation was disrupted due to damage of the nucleus basalis of Meynert (Murdoch et al., 2002). Compared to control brains, however, [³H]-nicotine binding sites measuring nAChRs were unaltered (Murdoch et al., 1998). Another report showed temporal cortical ChAT activity decreased by 50 percent in the postmortem brains of patients with fatal head injuries, but M1 and M2 mAChR binding was unaltered (Dewar and Graham, 1996).

In experimental traumatic brain injury, VAChT immunostaining was enhanced in the hippocampus and cortex, and M2 mAChR immunoreactivity was decreased in the

hippocampus one year following a 2.5 mm controlled cortical impact (CCI) (Dixon et al., 1999). Further studies showed this effect as early as two weeks in the hippocampus (Ciallella et al., 1998) with no change in mRNA (Shao et al., 1999). Binding of [³H]-vesamicol, indicative of VAChT, density was decreased as much as 50 percent immediately following CCI (Donat et al., 2008). Using a more diffuse model of TBI, fluid percussion resulted in memory deficits and decreases in ChAT activity in the dorsal hippocampus and frontal and temporal cortices one hour post injury (Gorman et al., 1996). However, this was accompanied by an increase in ChAT activity in the parietal cortex and a delayed increase in the medial septal area. Fluid percussion injury also caused a decrease in hippocampal M2 mAChR binding 24 hours following injury with no change in the M1 mAChR (DeAngelis et al., 1994). In contrast, enhanced binding of total mAChR sites was reported 15 days post injury in the hippocampus and neocortex (Jiang et al., 1994), but no change was found at one or 24 hours following injury and mAChR affinity was increased at one hour in the hippocampus (Lyeth et al., 1994). And most recently, mAChR was shown to be diminished at 24 and 72 hours following controlled cortical impact in various brain regions (Donat et al., 2008). Taken together, deficits in cholinergic neurotransmission occur following TBI presynaptically. Studies of the nAChR have been limited, but mAChRs show varying responses. It is possible that upregulation may be a result of compensatory mechanisms due to diminished ACh. Inconsistent findings for cholinergic receptors and other markers may be due to differences in experimental methodologies employed, extent of injury severity, brain region, and time period following TBI in which assessments were made.

Nicotinic AChR changes in experimental TBI

Previous studies from our laboratory have shown that experimental traumatic brain injury causes a widespread and significant loss of $\alpha 7$ nAChR binding in hippocampal and cortical brain regions (Verbois et al., 2000). $\alpha 7$ nAChR down-regulation occurs rapidly following TBI (within one hour), and persists for at least 21 days in some brain regions. Forty-eight hours after rats were subjected to a mild 1 mm CCI, there were significant decreases in multiple cortical and hippocampal brain regions in BTX binding, representing $\alpha 7$ nAChR density, in both the injured and uninjured sides

of the brain compared to sham-operated animals. Deficits were found in auditory cortex, CA1, CA2 and CA3 subfields of the hippocampus, dentate gyrus, stratum oriens, subiculum and superior colliculus. In rats receiving a moderate 2 mm injury, many regions on the contralateral side of the brain showed a significant decrease in $\alpha 7$ nAChR densities compared to sham, but hippocampal BTX binding was even further reduced on the injured side including some subfields demonstrating a 50 percent reduction compared to sham. Conversely, EPI binding, measuring non- $\alpha 7$ nAChRs, was not diminished and was found to be significantly elevated in the auditory cortex following mild or moderate TBI. In addition to the inconsistent effects on non- $\alpha 7$ nAChRs by region, enhanced mAChR binding, measured by [3 H]-quinuclidinyl benzilate (QNB), occurred following mild injury and reduced expression was noted following moderate injury. These results were less robust suggesting TBI related deficits were selective for $\alpha 7$ nAChRs.

In a time course study, 2 mm injury caused persistent deficits in $\alpha 7$ nAChR binding in the stratum oriens, lateral blade of the dentate gyrus, and CA2 subfield of the hippocampus at one hour following TBI through 21 days, the last time point tested (Verbois et al., 2002). Hippocampal CA1 deficits occurred at one hour but returned to baseline by the third day. Deficits in the subiculum and cortex occurred by one day but were transient only lasting 72 hours. The CA3 hippocampal subfield and superior colliculus showed deficits by one day and 72 hours, respectively, but continued during the 21 day period of testing. Deficits in EPI binding were delayed, with some regions showing transient changes in expression including the auditory cortex, a thalamic subregion, and the subiculum. Other regions such as the medial geniculate nucleus, dentate gyrus, superior colliculus, and another thalamic subregion were more persistent. We postulate nAChR deficits following experimental TBI may contribute to long term cognitive deficits in a variety of tests. Moreover, TBI is associated with cognitive impairment demonstrated in the Morris water maze task of learning and memory, and treatment with nicotine (Verbois et al., 2003b; Verbois et al., 2003a) attenuates this deficit, and nAChR selective ligands such as choline (Guseva et al., 2008) and others (unpublished data) may provide benefit. Thus, we hypothesize that $\alpha 7$ nAChRs are important mediators of cell death and survival pathways in the hippocampus and cortex,

and the pharmacological modulation of $\alpha 7$ nAChRs may be a promising treatment modality.

Galantamine as a possible pharmacotherapy in TBI

AChEIs used for the treatment of Alzheimer's disease, including galantamine, may have the potential to improve the cognitive dysfunction associated with traumatic brain injury and are the subject of ongoing research. Galantamine hydrobromide is marketed as Razadyne® ER (extended release) and Razadyne® (formerly Reminyl®) and is also available in generic form (Robinson and Plosker, 2006; Ortho-McNeil Neurologics, 2008). Galantamine is FDA approved to improve cognition and activities of daily living and slow the progression of cognitive symptoms in patients with mild to moderate Alzheimer's disease. Along with the NMDA receptor antagonist, memantine (Namenda®), galantamine and three other AChEIs, donepezil (Aricept®), rivastigmine (Exelon®), and tacrine (Cognex®) are the only FDA approved drugs employed for the treatment of AD. Tacrine was the first approved AChEI; however, its use is limited due to hepatotoxicity (Watkins et al., 1994; Cummings, 2004). In clinical trials, AChEIs demonstrate cognitive improvement compared to baseline while placebo groups show decline, with long term benefits in global function and cognition. In a one year study of donepezil treatment in AD, patients' cognitive status remained close to their baseline for one year while the placebo group significantly worsened (Winblad et al., 2001). In addition, AChEIs allow function and behavior to be maintained for longer periods and provide reductions in caregiver burden (Wilkinson et al., 2004). In one clinical trial studying galantamine, patients treated for three years experienced an 18-month period without cognitive decline (Raskind et al., 2004).

Galantamine (Robinson and Plosker, 2006; Ortho-McNeil Neurologics, 2008) is administered with an initial dose of 8 mg/day followed by a dose escalation after four week intervals of the previous dose, depending on patient tolerability up to 16-24 mg/day. Extended release galantamine (Razadyne® ER) is administered once daily (in the morning with food) while the daily dose of the immediate release form of galantamine is divided twice daily. An oral solution is also available. Adverse reactions may include nausea, vomiting, dizziness, depression, anorexia, and weight loss.

Galantamine has an elimination half-life of 7-8 hours; it undergoes hepatic metabolism by cytochrome P450 (CYP) isoforms CYP2D6 and CYP3A4, and is also glucuronidated and excreted unchanged in the urine.

Both clinical and experimental investigations have demonstrated a disruption in cholinergic neurotransmission following TBI. For example, the cognitive sequelae of TBI has been linked to the basal forebrain (Salmond et al., 2005). In this study, imaging was performed by MRI scans and voxel-based morphometry, a technique that was used to detect decreases in grey matter density in the basal forebrain region containing ACh cell bodies and in cortical and hippocampal regions containing ACh projections. Attempts have been made to treat the cognitive sequelae of TBI with cholinergic enhancing drugs. Patients with AD and TBI may exhibit similar memory dysfunction and other cognitive impairments. There are several reports of the clinical evaluation (clinical trials or clinical experience) of AChEIs following TBI, most of which have been conducted with donepezil (Aricept®), tacrine, or physostigmine resulting in cognitive improvement (Levin et al., 1986; Cardenas et al., 1994; Pike et al., 1997; Taverni et al., 1998; Masanic et al., 2001; Zhang et al., 2004; Khateb et al., 2005). In one report following AChEI use in patients with TBI related chronic impairments including target symptoms such as poor memory, patients treated with long term galantamine for 6-33 months as well as those treated with other AChEIs expressed benefits in functioning (Tenovuo, 2005). Furthermore, one case report showed galantamine in combination with the atypical antipsychotic, risperidone, improved cognitive deficits in a patient experiencing schizophrenia-like psychosis as a result of severe TBI (Bennouna et al., 2005). However, according to another case report, galantamine was not beneficial in improving cognitive and behavioral symptoms following a severe TBI (Johnson et al., 2007).

In animal models, galantamine induces upregulation of nAChRs. Galantamine administration caused an upregulation in [³H]-cytisine binding in hippocampus and [¹²⁵I]-BTX binding in the cortex of FVB/N (control) mice (Svedberg et al., 2004). Rabbits treated with galantamine also showed enhanced nAChR expression and improved learning and memory (Woodruff-Pak and Santos, 2000; Woodruff-Pak et al., 2001). Galantamine has also been shown to reverse cognitive impairment caused by the non-competitive nAChR antagonist, mecamylamine (Woodruff-Pak et al., 2003).

The efficacy of AChEIs does not necessarily correlate with the degree of enzyme inhibition. Galantamine is a long acting, centrally active, selective, competitive and reversible AChEI. While galantamine is only a modest AChEI, it acts as an allosteric potentiating ligand for the nicotinic receptor, and studies have shown that galantamine may elicit a neuroprotective effect through its actions on nAChRs (Coyle et al., 2007; Wang et al., 2007). While donepezil (Taverni et al., 1998; Khateb et al., 2005) has demonstrated improvements in cognitive measures, the strong APL activity of galantamine suggests its ability to enhance nicotinic receptor activation is involved in its neuroprotective effects.

Allosteric potentiating ligands (APLs) (Schrattenholz et al., 1996; Maelicke et al., 2001), which are also known as positive allosteric modulators (PAMs) (Faghieh et al., 2007), of the nicotinic receptor are sensitizing agents that elicit their actions through interactions with a binding site other than the classical pharmacophore upon which ACh, and other antagonists and agonists, are thought to bind (Pereira et al., 1993; Pereira et al., 1994; Schrattenholz et al., 1996). Exposure to these drugs alone does not cause receptor desensitization or compensatory changes in neurotransmitter expression. There are both positive and negative APL sites located on the α subunit of nAChRs that modulate channel opening and ion conductance (Pereira et al., 1993). The AChEIs, physostigmine, tacrine, and galantamine all display positive APL activity (Svensson and Nordberg, 1996). Electrophysiological studies show galantamine is an APL at both $\alpha 4\beta 2$ nAChRs (Samochocki et al., 2000) and $\alpha 7$ nAChRs (Maelicke et al., 2001).

APLs may act by sensitizing nicotinic receptors by producing enhanced channel opening. In electrophysiological studies of human embryonic kidney cells expressing human $\alpha 4\beta 2$ nAChR, galantamine potentiates acetylcholine mediated whole-cell responses (increased peak amplitude). Galantamine also shifts the dose response curve to the left thereby enhancing affinity to $\alpha 4\beta 2$, and in a cell line expressing chimeric $\alpha 7$ this was evident to a greater degree for $\alpha 7$ (Maelicke et al., 2001). Galantamine acts as an APL at lower concentrations (0.8 μ M -5 μ M) and at higher concentrations is a direct channel blocker like other AChEIs, specifically tacrine (Maelicke et al., 2001). APLs facilitate synaptic neurotransmission (Santos et al., 2002). Galantamine enhanced nAChR mediated GABAergic and glutamatergic transmission in rat hippocampal and

human cortical slices (Santos et al., 2002), and at a very specific time point (3 hour pretreatment) in rats galantamine can enhance nicotine mediated, norepinephrine release in the hippocampus (involving cognition) but not dopamine from the nucleus accumbens (Sharp et al., 2004). And recently, galantamine has been shown to attenuate the cognitive deficits caused by the intracerebroventricular injection of A β in mice due to galantamine's APL actions resulting in enhanced dopamine neurotransmission (Wang et al., 2007).

The neuroprotective properties of galantamine treatment have been demonstrated in several *in vitro* models. Galantamine prevented neurotoxicity in oxygen and glucose deprived rat hippocampal slices as measured by lactate dehydrogenase release when given before and after (Sobrado et al., 2004) the insult. Galantamine treatment of SH-SY5Y human neuroblastoma cells and bovine chromaffin cells both expressing nAChRs (Arias et al., 2004) protected cells against soluble A β and the Ca²⁺ depleting, thapsigargin, both causing apoptotic induced toxicity. This effect was reduced by BTX and caused upregulation of α 7 receptors and expression of the anti-apoptotic protein, Bcl-2. By contrast, tacrine was not neuroprotective (Arias et al., 2004). In fetal rat cortical primary cultures, galantamine has been shown to ameliorate neurotoxicity produced by the cotreatment of glutamate and A β (which enhances glutamate toxicity), and this was partially blocked by the α 7 nAChR antagonists, MLA and BTX but not the α 4 β 2 receptor antagonists, DH β E, suggesting an allosteric site. Galantamine was also shown to enhance the neuroprotective effect of nicotine against glutamate induced toxicity in a study in which either treatment alone did not (Kihara et al., 2004). The authors concluded the response was due to actions on nAChR downstream signaling cascades involved with phosphatidylinositol 3-kinase (PI3K) directly or through promoting nicotine's effect on nAChRs.

Due to the combination of AChEI activity and APL properties at nAChRs, the study of galantamine in a model of TBI may better characterize galantamine's modulatory properties at nAChRs and potential cognitive benefit. The second research question is then addressed in the dissertation: Does galantamine, a commonly used pharmacotherapy for functional improvement in AD patients, target nicotinic receptor deficits to improve cognitive impairment following experimental brain injury?

The regulation of nAChRs is of particular interest in that pharmacological modulation of receptor density does not involve transcriptional mechanisms. For example, when mice were chronically treated with nicotine resulting in nAChR upregulation throughout the brain demonstrated by [³H]-nicotine binding (Marks et al., 1983; Marks et al., 1985; Marks et al., 1992; Robinson et al., 1996), there was no upregulation of $\alpha 4$ or $\beta 2$ mRNA. Chronic corticosterone treatment caused downregulation of the $\alpha 7$ nAChR in mice as demonstrated by [¹²⁵I]-BTX binding (Pauly and Collins, 1993; Robinson et al., 1996), and this occurred with no change in mRNA (Pauly and Collins, unpublished data). In Alzheimer's disease, decrements in nAChRs do not appear to correlate with deficits in mRNA expression. Thus, the third research question addressed in the dissertation is: Are $\alpha 7$ nicotinic acetylcholine receptor protein deficits accompanied by deficits in mRNA expression following experimental traumatic brain injury?

Statement of the Research Problem and Research Plan

Neuronal nicotinic cholinergic receptors (nAChRs) are important mediators of cognitive function including learning and memory processes in the brain (Levin and Simon, 1998; Paterson and Nordberg, 2000). The first reports of deficient cholinergic activity in autopsy brains of patients with Alzheimer's disease (AD) related cognitive impairment (Davies and Maloney, 1976; Perry et al., 1978) occurred over thirty years ago and led to "the cholinergic hypothesis of geriatric memory dysfunction" described by Bartus soon thereafter (Bartus et al., 1982). Since then, extensive research has been conducted in order to understand the role of the cholinergic system in learning and memory in healthy individuals as well as the role of an altered cholinergic system in diseases involving cognitive impairment.

AD is a progressive neurodegenerative disease resulting in memory loss, diminished cognition, and neurobehavioral disturbances. A deficit in cholinergic neurotransmission, particularly involving the downregulation of nAChR density (Pauly, 1999; Court et al., 2001; Nordberg, 2001), is associated with the symptomatology of Alzheimer's disease, and correcting the cholinergic deficit has been the focus of current therapeutics and ongoing research. Similarly, traumatic brain injury (TBI) may involve persistent neuropsychiatric sequelae characterized by impairments in cognition (Arciniegas, 2003). Investigations have revealed a dysfunction of cholinergic neurotransmission following fatal traumatic brain injury (Murdoch et al., 1998) and in individuals with cognitive sequelae related to a past brain injury (Salmond et al., 2005).

Using experimental models of Alzheimer's disease and traumatic brain injury as paradigms for neurodegeneration, the research herein seeks to evaluate changes in nicotinic cholinergic receptors in both disease states, understand how these changes are regulated, and investigate how treatment with a modulator of nicotinic receptors affects these changes. A summary of AD and TBI similarities and the models employed in the dissertation research is found in table 1.3. The following research questions are addressed in three studies included in the dissertation:

1. Is the $\alpha 7$ nicotinic acetylcholine receptor an early marker in experimental Alzheimer's disease?

2. Does galantamine, a commonly used pharmacotherapy for functional improvement in AD patients, target nicotinic receptor deficits and improve cognitive impairment following experimental brain injury?
3. Are $\alpha 7$ nicotinic acetylcholine receptor protein deficits accompanied by deficits in messenger RNA (mRNA) expression following experimental traumatic brain injury?

Hypotheses and Specific Aims

The purpose of this dissertation was to investigate changes in $\alpha 7$ nAChR expression in models of neurodegenerative disease and test the hypothesis that allosteric modulators of the $\alpha 7$ nAChR exert neuroprotective properties or enhance functional recovery following experimental brain injury. The experimental models employed include a transgenic mouse model of Alzheimer's disease (APPswe mice) and the controlled cortical impact injury model of TBI in rats. Three specific hypotheses were evaluated.

Hypothesis 1: Young APPswe mice display a significant upregulation in $\alpha 7$ nicotinic receptor expression throughout the brain as a result of A β functioning as an agonist at the $\alpha 7$ nAChR, while aged APPswe mice demonstrate reduced expression.

Specific Aim 1: To investigate the effects of aging in APPswe and wild-type mice on $\alpha 7$ nAChRs as measured by BTX binding

Specific Aim 2: To compare alterations in BTX binding in APPswe and wild-type mice with non- $\alpha 7$ nAChRs and non-nicotinic receptors

Hypothesis 2: Galantamine, an acetylcholinesterase inhibitor and allosteric potentiating ligand for nAChRs, facilitates neuroprotection and cognitive enhancement in an experimental model of traumatic brain injury

Specific Aim 1: To evaluate the effects of the nicotinic receptor modulator, galantamine, on acquisition and retention tests using the Morris water maze task of spatial memory following TBI

Specific Aim 2: To investigate the neuroprotective effects of galantamine as evidenced by cortical tissue sparing following TBI

Specific Aim 3: To characterize galantamine mediated alterations in $\alpha 7$ nAChR receptor expression using quantitative nicotinic receptor autoradiography in experimental TBI

Hypothesis 3: Deficits in $\alpha 7$ nAChR expression two days following experimental traumatic brain injury are due to reduced expression of $\alpha 7$ nAChR mRNA

Specific Aim 1: To determine if decrements in $\alpha 7$ nAChR receptor expression as measured by BTX binding correlate with changes in mRNA expression measured by *in situ* hybridization following experimental TBI

Specific Aim 2: To compare the changes in $\alpha 7$ nAChRs following TBI with the expression and regulation of non- $\alpha 7$ nAChRs

Table 1.1. A summary of the nicotinic cholinergic receptor changes in AD measured with radioligands or by immunological detection.

Detection Method	Subtype Selectivity	Regional Changes in AD	References
Radioligand			
³ H-Nicotine	Non-selective agonist (non- α 7)	↓ temporal, infratemporal, frontal, parietal, occipital CTX, Hipp (including dentate granular layer, stratum lacunosum), entorhinal CTX, presubiculum, parahippocampal gyrus, nbM, caudate, putamen; ↔ frontal CTX, hippocampal CA1/2 (stratum lacunosum), CA2/3, dentate gyrus, subiculum	(Flynn and Mash, 1986; Shimohama et al., 1986; Whitehouse et al., 1986; Whitehouse et al., 1988b; Whitehouse et al., 1988a; Rinne et al., 1991; Aubert et al., 1992; Perry et al., 1995; Warpman and Nordberg, 1995; Hellstrom-Lindahl et al., 1999; Marutle et al., 1999; Sihver et al., 1999; Perry et al., 2000)
³ H-Epipatidine	Non- α 7 agonist	↓ temporal, frontal, parietal, occipital CTX, Hipp, thalamus, putamen	(Warpman and Nordberg, 1995; Hellstrom-Lindahl et al., 1999; Martin-Ruiz et al., 1999; Marutle et al., 1999; Sihver et al., 1999; Perry et al., 2000)
³ H-Cytisine	β 2 agonist (α 4 β 2)	↓ temporal CTX	(Sihver et al., 1999)
ABT-418	α 4 β 2 agonist	↓ temporal CTX	(Warpman and Nordberg, 1995)
5- ¹²⁵ I-A-85380	α 4 β 2 agonist	↓ entorhinal CTX, caudate; ↔ thalamus	(Pimlott et al., 2004)
³ H-N-methyl-carbamylocholine	Non-selective agonist	↓ frontal, temporal, parietal, occipital CTX, Hipp; ↔ subcortical (striatum, globus pallidus, thalamus, nbM) regions	(Araujo et al., 1988)
¹²⁵ I- α -bungarotoxin	α 7 antagonist	↓ Hipp; ↔ entorhinal CTX, hippocampal subfields (stratum lacunosum, CA2/3, dentate gyrus), subiculum	(Hellstrom-Lindahl et al., 1999; Perry et al., 2000)

Continued

Table 1.1 (Continued)

Detection Method	Subtype Selectivity	Regional Changes in AD	References
<u>Immunological</u>			
α 4 subunit protein		↓ frontal, temporal CTX, entorhinal CTX, Hipp	(Sparks et al., 1998; Martin-Ruiz et al., 1999; Wevers et al., 1999; Burghaus et al., 2000; Guan et al., 2000; Perry et al., 2000; Wevers et al., 2000; Yu et al., 2003; Teaktong et al., 2004; Yu et al., 2005)
β 2 subunit protein		↓, ↔ temporal CTX; ↔ Hipp	(Sparks et al., 1998; Guan et al., 2000)
α 7 subunit protein		↓ frontal, temporal CTX, Hipp; ↑ entorhinal cortex, subiculum, and some hippocampal subfields (CA3/4 and stratum granulosum); ↔ temporal CTX	(Martin-Ruiz et al., 1999; Wevers et al., 1999; Banerjee et al., 2000; Burghaus et al., 2000; Guan et al., 2000; Wevers et al., 2000; Teaktong et al., 2004; Yu et al., 2005)
α 3 subunit protein		↓, ↔ temporal CTX; ↓ Hipp	(Martin-Ruiz et al., 1999; Guan et al., 2000)
<u>mRNA</u>			
α 4		↔ CTX, Hipp	(Hellstrom-Lindahl et al., 1999; Wevers et al., 1999; Wevers et al., 2000)
α 7		↔ CTX, temporal CTX; ↑ Hipp, nbM	(Hellstrom-Lindahl et al., 1999; Wevers et al., 1999; Wevers et al., 2000; Counts et al., 2007)
α 3		↔ temporal CTX, entorhinal CTX, Hipp, thalamus	(Terzano et al., 1998; Hellstrom-Lindahl et al., 1999)

Abbreviations: CTX, (cerebral) cortex; Hipp, hippocampus; nbM, nucleus basalis of Meynert; ↓, decrease; ↔, no change; ↑, increase

Table 1.2. A summary of the muscarinic cholinergic receptor changes in AD measured with radioligands or by immunological detection.

Detection Method	Regional Changes in AD	References
<u>Radioligand Selectivity*</u>		
M3	↓ entorhinal CTX, Hipp ↔ CTX, nbM, Hipp, striatum	(Flynn et al., 1995; Rodriguez-Puertas et al., 1997)
M1-M5	↓ CTX, frontal, visual CTX, Hipp, nbM, entorhinal CTX	(Shimohama et al., 1986; Rodriguez-Puertas et al., 1997)
M1	↓ frontal, visual CTX entorhinal CTX, Hipp; ↔ CTX, Hipp; ↑ Hipp, striatum	(Mash et al., 1985; Araujo et al., 1988; Aubert et al., 1992; Rodriguez-Puertas et al., 1997)
M2	↓ CTX, Hipp; ↔ subcortical regions; ↑ striatum	(Mash et al., 1985; Araujo et al., 1988; Aubert et al., 1992; Rodriguez-Puertas et al., 1997)
<u>Immunological</u>		
M1	↓ CTX, Hipp	(Flynn et al., 1995)
M2	↓ CTX, Hipp; ↔ nbM	(Flynn et al., 1995; Mufson et al., 1998)
M4	↑ CTX; ↔ CTX, Hipp, striatum	(Flynn et al., 1995; Rodriguez-Puertas et al., 1997)
M5	↔ CTX, subcortical regions	(Flynn et al., 1995)
<u>mRNA</u>		
M1	↓ temporal CTX; ↑ temporal CTX	(Harrison et al., 1991; Wang et al., 1992)
M2	↔ temporal CTX	(Wang et al., 1992)
M3	↔ temporal CTX	(Wang et al., 1992)
M4	↔ temporal CTX	(Wang et al., 1992)

* Studies employed the use of multiple radioligands, as this method is needed to measure some muscarinic subtypes selectively
Abbreviations: CTX, (cerebral) cortex; Hipp, hippocampus; nbM, nucleus basalis of Meynert; ↓, decrease; ↔, no change; ↑, increase

Table 1.3. The study of Alzheimer’s disease and traumatic brain injury as paradigms for neurodegenerative disease: a review of the similarities and models employed in the dissertation research.

Alzheimer’s Disease	Traumatic Brain Injury
Similar pathophysiological mechanisms: excitotoxicity (chronic in AD, acute in TBI), inflammation, mitochondrial damage, and oxidative stress	
Parallel cognitive impairments: cholinergic (nicotinic in AD) and glutamatergic system dysfunction leads to alterations in learning, memory, and behavior	
Concurrent neuropathologies:	
<ul style="list-style-type: none"> • Soluble oligomeric Aβ, neuritic plaques and neurofibrillary tangles 	<ul style="list-style-type: none"> • May result in APP and Aβ accumulation in axons, diffuse Aβ deposits, neurofibrillary tangles
Other relevant comparisons:	
<ul style="list-style-type: none"> • Age related in most cases • Apolipoprotein E4 allele increases the risk of developing AD • MCI may develop into AD 	<ul style="list-style-type: none"> • All ages, age may increase the risk of AD related neuropathologies • Repetitive mild TBI accelerates neuropathology in a preclinical model • Apolipoprotein E4 increases the risk of Aβ related neuropathology and AD • NFL players with repeated mild TBI have an increased risk for MCI and AD • Athletes may develop behavioral and cognitive symptoms following mild TBI, known as CTE, displaying brain atrophy and neurofibrillary tangles
Models used to conduct the dissertation research:	
<ul style="list-style-type: none"> • APP^{swe} mice, a transgenic mouse model overexpressing APP • Large age range examined (models can vary in severity based on onset of pathology) • AD pathophysiology targets selective areas of the brain but may better correspond to a diffuse injury • Disease process is chronic 	<ul style="list-style-type: none"> • CCI injury in rats resulting in contusion • mild and moderate injury severities examined • CCI is representative of a focal injury • Disease process is acute to chronic
Abbreviations: AD, Alzheimer’s disease; TBI, traumatic brain injury; A β , beta-amyloid peptide; APP, amyloid precursor protein; MCI, mild cognitive impairment; CTE, chronic traumatic encephalopathy; APP ^{swe} , APP mice expressing the Swedish double mutation; CCI, controlled cortical impact	

Chapter 2: Upregulation of Hippocampal $\alpha 7$ nAChRs in a Transgenic Mouse Model of Alzheimer's Disease

Introduction

Alzheimer's disease (AD) is an age related, progressive neurodegenerative disease (Alzheimer's Association, 2009). AD is the most common form of dementia and involves memory loss, cognitive dysfunction, and alterations in behavior and personality that ultimately disrupt or prevent the activities of daily living. As of 2009, it was estimated that 5.3 million Americans were living with AD, and the prevalence is expected to rise considerably as the population ages (NIA, 2005; Alzheimer's Association, 2009).

Synaptic loss is an important characteristic defining Alzheimer's disease (Selkoe, 2002). Two neuropathological hallmarks are implicated in the pathogenesis of AD, neuritic plaques and neurofibrillary tangles (Hardy and Selkoe, 2002; Selkoe, 2005). Extracellular neuritic plaques are made up of dystrophic neurons surrounding a dense core containing the β -amyloid ($A\beta$) peptide. Intracellular neurofibrillary tangles contain the hyperphosphorylated form of the microtubule protein, tau (Hardy and Selkoe, 2002; Selkoe, 2005). $A\beta$ is formed by enzymatic cleavage of the amyloid precursor protein (APP). APP undergoes cleavage through the actions of β - and γ -secretase to form $A\beta 40$ and $A\beta 42/43$, to a lesser extent; moreover, mutations in APP or the presenilins, the catalytic component of γ -secretase, lead to increased formation of the larger peptide, $A\beta 42/43$, involved in self-aggregation, oligomerization, and plaque formation (Blennow et al., 2006; Walsh and Selkoe, 2007; Querfurth and LaFerla, 2010). Five percent of patients with AD have an inherited form of the disease known as Familial Alzheimer's disease (FAD) (St George-Hyslop, 2000). In FAD, patients present with an earlier, more aggressive course caused by the presence of mutations in the amyloid precursor protein (APP) or the presenilins (PS1, PS2), both resulting in altered forms of $A\beta$, including the Swedish double mutation (K670N, M671L, often referred to as APP_{swe}) in the APP gene (Mullan et al., 1992).

Postmortem research has demonstrated that Alzheimer's disease is associated with degeneration of basal forebrain cholinergic neurons resulting in the loss of cholinergic innervation to the cortex and hippocampus (Davies and Maloney, 1976; Perry et al., 1978) suggesting that cognitive impairment seen in AD patients is correlated with deficits in CNS cholinergic neurotransmission (Bartus et al., 1982; Bartus, 2000).

Studies of postmortem AD tissue also demonstrate changes in nicotinic cholinergic receptor expression. Reductions in nicotinic acetylcholine receptor (nAChR) densities measured by radioligand binding using nicotine, epibatidine (EPI), cytisine, the latter two selective for $\alpha 4\beta 2$ subtypes, and others are consistently documented in AD in several cortical and hippocampal regions (Flynn and Mash, 1986; Whitehouse et al., 1986; Whitehouse et al., 1988b; Whitehouse et al., 1988a; Rinne et al., 1991; Aubert et al., 1992; Perry et al., 1995; Warpman and Nordberg, 1995; Hellstrom-Lindahl et al., 1999; Martin-Ruiz et al., 1999; Marutle et al., 1999; Sihver et al., 1999; Perry et al., 2000). Furthermore, *in vivo* nicotine binding assessed by PET has demonstrated reductions in nAChR densities in the cerebral cortex and hippocampus of AD patients (Nordberg et al., 1990; Nordberg et al., 1995; Nordberg et al., 1997).

However, studies of mild cognitive impairment (MCI) (Gilmor et al., 1999; DeKosky et al., 2002) and early AD suggest a sparing of cholinergic neurotransmission. No loss of nAChRs was detected through PET scanning of patients with early AD (Ellis et al., 2008). Interestingly, $\alpha 7$ nAChRs, as measured by [3 H]-methyllycaconitine (MLA), demonstrated a non-significant elevation in patients with mild to moderate AD compared to patients with MCI or patients without cognitive impairment. Patients with whom a diagnosis of AD was confirmed by neuropathological analysis demonstrated elevated MLA binding and A β levels (Ikonovic et al., 2009).

Changes in cholinergic neurotransmission have been demonstrated in transgenic mouse models of AD containing APP mutations such as APP^{swe} alone (most notably the Tg2576 model) or in combination with PS1 mutations. Several studies have reported reductions in nAChRs. Tg2576 mice demonstrated a reduction in cortical [3 H]-cytisine binding in aged mice (Apelt et al., 2002); however, young mice prior to A β deposition showed a downregulation of cortical $\alpha 4$ nAChR mRNA, but no change in the mRNA of $\alpha 7$ or $\alpha 3$ receptor subunits (Mousavi et al., 2004).

In mice expressing APP_{swe}, PS1 and a tau mutation (3xTg-AD), decreased BTX binding was detected at an age showing intraneuronal accumulation of A β 42 but prior to A β deposition in the cortex, hippocampus, and thalamus (Oddo et al., 2003), and EPI binding was upregulated in the thalamus (Oddo et al., 2005). A study including multiple ages and the progression of A β pathology showed no change in α 7 or α 4 β 2 nAChRs, as measured by BTX and cytosine binding, respectively, in the parietal cortex and hippocampus of three week to 17 month old APP_{swe} and PS1 double transgenic mice (Marutle et al., 2002).

Recent studies have found an elevation in α 7 nAChR expression in Tg2576 mice in the hippocampus and dentate gyrus with age, beginning at four months in the dentate, compared to wild-type mice, but no change in α 4 nAChR expression (Dineley et al., 2001). Further analysis showed upregulated hippocampal α 7 nAChR protein expression in five month old Tg2576 mice, prior to A β deposition, that accompanied deficits in the contextual fear learning behavioral task indicating hippocampal impairment (Dineley et al., 2002b). Furthermore, an increase in α 7 nAChRs as measured by BTX binding was demonstrated in the cerebral cortex, hippocampus, caudate, and cerebellum of four month old Tg2576 mice preceding A β plaque formation and behavioral changes. A similar upregulation was found in α 4 β 2 nAChRs as measured by cytosine binding but only in older mice, and no change was found in mAChR binding. Enhanced nAChR binding occurred along with an upregulation in the mRNA levels of both α 7 and α 4 receptor subunits (Bednar et al., 2002). In addition, older Tg2576 mice, aged 14.5 months, displayed increased BTX binding in cortex compared to wild-type mice (Hellstrom-Lindahl et al., 2004b). An electrophysiologic analysis showed transgenic models overexpressing A β retain functional α 7 nAChRs (Howlett et al., 2004; Spencer et al., 2005).

Along with the evidence that the nAChR may be differentially affected in early and late stage disease, a selective interaction between the nAChR and A β has been well documented. Furthermore, data suggest that A β binds with high affinity to α 7 nAChRs (Wang et al., 2000b; Wang et al., 2000a). There are conflicting reports regarding the pharmacologic properties of A β at this receptor and the physiologic consequences of this

interaction (Dineley et al., 2001; Liu et al., 2001; Pettit et al., 2001; Vaucher et al., 2001; Dineley et al., 2002a; Tozaki et al., 2002; Dougherty et al., 2003; Grassi et al., 2003; Lee and Wang, 2003; Li and Buccafusco, 2004; Wu et al., 2004; Spencer et al., 2005).

However, several studies suggest A β displays agonist properties at the nAChR, and these studies are reviewed in the following references (Dineley, 2007; Jurgensen and Ferreira, 2010). There is evidence that A β 's pharmacological properties are related to concentrations and disease progression, as the agonist behavior appears to be an early event (Hernandez et al., 2010).

Combining the findings suggesting a sparing of cholinergic markers in MCI, the recent report of the preservation of nAChRs *in vivo* in early AD, the data indicating an early elevation in nAChR expression in AD mouse models, and the data demonstrating the interaction of $\alpha 7$ nAChRs and A β , the $\alpha 7$ nAChR may be an early target in Alzheimer's disease. Upregulation of presynaptic markers and nAChR may indicate short term compensation to help combat cognitive impairments. Our laboratory was interested in determining if receptor changes occur as an early event prior to the development of neuropathology or if receptor alterations occur as a late consequence in experimental Alzheimer's disease. We hypothesize that young APP^{swe} mice display a significant upregulation in $\alpha 7$ nAChR throughout the brain as a result of A β functioning as an agonist at the $\alpha 7$ nAChR. Most investigators have limited the study of cholinergic receptor expression to cortical and hippocampal regions. Our study was designed to evaluate the expression of nAChRs in brain regions throughout the brain in a mouse model of Alzheimer's disease, which has never before been tested for cholinergic receptor indices. Our results suggest that $\alpha 7$ changes in transgenic mice overexpressing APP are significantly more complex than previously suggested.

Materials and Methods

Animals and tissue preparation

This longitudinal study of aging was performed using 45 to 558 day (approximately 1.5-18 months) old male mice. Male mice were used to avoid any male or female related differences due to sex hormones. The mice included 24 transgenic mice

(APP^{swe}) expressing chimeric mouse/human APP 695 containing the Swedish double mutation (Lys 670 → Asn and Met 671 → Leu [using the APP 770 numbering]) and 24 wild-type (B6C3F1) controls developed by Borchelt et al. (Borchelt et al., 1996). APP^{swe} mice ranged in age from 45 to 533 days and wild-type mice ranged in age from 45 to 558 days. The mice were generously provided by Dr. Mark Mattson of the Laboratory of Neurosciences, National Institute on Aging. All mice were housed in facilities at the National Institutes on Aging and were euthanatized over a two day period. Brains were excised, frozen in isopentane, and placed on dry ice for shipment to the University of Kentucky where they were stored at -80°C until further processing. Brains were then sectioned 16 μm thick using a Leica CM50 cryostat (Nussloch, Germany) and were mounted onto slides coated with gelatin, chromium potassium sulfate and poly-L-lysine in order to promote tissue adherence for autoradiographic analysis. For immunohistochemistry, additional sections were collected on Superfrost Plus® slides (Fisher Scientific, Pittsburgh, PA) containing two sections per slide. Sections were collected throughout the entire rostro-caudal axis of the mouse brain, beginning at approximately Figure 19 (interaural 5.22 mm, bregma 1.42 mm) and ending at approximately Figure 78 (interaural -1.88 mm, bregma -5.68 mm) according to Paxinos and Franklin's mouse brain atlas (Paxinos and Franklin, 2001). Serial sets of adjacent tissue sections were obtained in order to evaluate the binding of multiple radioligands in specific anatomical regions throughout the brain. Brain sections were stored at -80°C until use at which time they were thawed and air dried prior to experimentation. All reagents were purchased from either Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) unless otherwise noted.

Cholinergic receptor autoradiography and image analysis

Quantitative receptor autoradiography was performed to investigate cholinergic receptor binding in APP^{swe} and wild-type mice. All radioligands were purchased from PerkinElmer Life Sciences, Inc., Boston, MA. Nicotinic cholinergic receptor autoradiography was employed using the radioligands, α -[¹²⁵I]-bungarotoxin and [¹²⁵I]-epibatidine to measure $\alpha 7$ and non- $\alpha 7$ nAChR binding, respectively. α -[¹²⁵I]-bungarotoxin (BTX) binding was carried out as previously described by Pauly and

Collins (Pauly and Collins, 1993). The concentration of radioligand used was 2-3 fold greater than the affinity (K_d) for binding to the receptor to ensure the assay results are representative of changes in receptor number (B_{max}) as opposed to alterations in affinity. In detail, brain sections were first pre-incubated in Krebs-Ringer HEPES (KRH) buffer, pH 7.5 (1180 mM NaCl, 48 mM KCl, 25 mM $CaCl_2$, 12 mM $MgSO_4(7H_2O)$, 200 mM HEPES, and 100 mM NaOH) for 30 minutes at room temperature. Next, 2.5 nM α - $[^{125}I]$ -BTX (specific activity = 18 $\mu Ci/\mu g$) was added to KRH buffer containing 0.5 mg/ml bovine serum albumin, to protect the radioligand from proteases and limit adherence to plastic or glass, in which sections were incubated for two hours at room temperature. This was followed by a series of washes (three x 20 minutes in KRH, one x 10 seconds in 10% KRH, and one x 10 seconds in double deionized water [ddH₂O]) at 4°C.

Non- $\alpha 7$ nAChR binding was determined through the use of $[^{125}I]$ -epibatidine (EPI), specific activity = 2200 Ci/mmol, according to the original method of Perry and Kellar in which tritiated EPI was employed (Perry and Kellar, 1995). Due to the unavailability of tritium sensitive film at the time of experimentation, the iodinated radioisotope was used instead which provided an added advantage in that the exposure time was reduced from months to days. $[^{125}I]$ -EPI possesses similar properties to the tritiated form such as a high affinity and low non-specific binding (Davila-Garcia et al., 1997; Whiteaker et al., 2000). However, due to a significantly higher specific activity of the iodinated form and limited resources, it was not possible to purchase the radioligand in sufficient concentration to conduct assays at saturation, so a concentration of EPI in the low pM range was used. Brain sections were incubated with EPI in a pH 7.0 buffer containing 50 mM Tris HCl, 120 mM NaCl, 5 mM $MgCl_2$, and 2.5 mM $CaCl_2$ for 40 minutes at room temperature. The incubation was followed by a number of washes at 4°C (two x 5 minutes in buffer, one x 10 seconds in 10% buffer, and one x 10 seconds in ddH₂O).

Muscarinic cholinergic receptor autoradiography was performed using the radioligand, $[^3H]$ -quinuclidinyl benzilate (QNB; specific activity = 39 Ci/mmol) as previously described (Pauly et al., 1989; Verbois et al., 2000). QNB binds to total (M1-M5) mAChRs and a concentration of 1nM was used so that binding reflected a measure of total receptor number. First, sections were pre-incubated in 50 mM Tris HCl at pH 7.4

buffer for 15 minutes at 4°C followed by an incubation containing QNB and pre-incubation buffer for 90 minutes at room temperature. The experiment was completely with several washes at 4°C (three x 4 minutes in buffer, one x 10 seconds in 10% buffer, and one x 10 seconds in ddH₂O).

Once the washes were completed, sections were dried under a low speed fan and stored overnight under vacuum in a desiccator at room temperature. The following day, sections from the BTX and EPI binding assays were exposed to Hyperfilm-βmax film and the QNB assay to Hyperfilm-³H film (Amersham, Arlington Heights, IL). Exposures were stored in x-ray cassettes along with slides containing calibrated brain tissue paste standards with known amounts of either ¹²⁵I or ³H depending on the radioligand used. Tissue standards were prepared as described elsewhere (Geary et al., 1985; Pauly and Collins, 1993). Exposure times were 10 days and three days for the BTX, and QNB binding assays, respectively. The EPI binding assay was exposed for 48 hours for all brain regions except the dentate which was exposed for seven days and processed with Kodak D-19 developer (Eastman Kodak Co., Rochester, NY).

Brain images were digitized for quantification using a Power Macintosh 950 and NIH Image version 1.59 software, a Scion LG-3 frame grader/imaging board (Scion Corp, Baltimore, MD), Sony XC-77 CCD video camera (Sony, Towada, Japan), and a Northern Lights desktop illuminator (Model B95 Imaging Research, Ontario, Canada). A tissue radioactivity versus optical density standard curve, fit to a third degree polynomial, or other best fit model, was generated to determine molar quantities of bound ligand, expressed as nCi/mg of wet tissue weight, from measured optical densities of the brain regions of interest. Each structure was measured bilaterally in multiple sections. Previous studies investigating cholinergic receptor alterations in transgenic models of AD have typically focused on cortical and hippocampal regions due to the impact of AD pathology on these regions. The present study evaluated changes in cholinergic receptor expression throughout the rostral-caudal axis of the brain beginning at regions just anterior to the level of the striatum and ending in the pons.

A β histochemistry

Two approaches were used to evaluate A β deposition in the brains of animals used in this study. Immunohistochemistry and thioflavin-S staining were performed to determine the presence and localization of A β protein deposits with relation to age in both transgenic and wild-type mice. Immunohistochemistry was performed employing a modified version of the original methods presented by Hyman et al. (Hyman et al., 1992) using the mouse monoclonal human A β protein clone, 4G8, that reacts to the human A β peptide at amino acid residues 17-24 (Signet Laboratories, Inc. Dedham MA). Briefly, reagents were applied to slides containing two brain slices each surrounded by a hydrophobic barrier using a PAP pen slide marker (Research Products International Corp., Mt. Prospect, IL). Thawed sections were first fixed in 4% paraformaldehyde for 10 minutes and rinsed fully in water. Paraformaldehyde (4%) was made from 16% (Electron Microscopy Sciences, Ft. Washington, PA) diluted first 1:1 in ddH₂O with the resulting 8% solution diluted further in equal parts 0.2 M Sorensen's Phosphate buffer (37.74g Na₂HPO₄(7H₂O), 8.06g KH₂PO₄ q.s. 1L ddH₂O). Next the following treatments were applied with ddH₂O rinses in between each: 0.3% TBS (50mM Tris-ultra pure and 150 mM NaCl, pH 7.4) containing 0.1% Triton-X (TTBS) for 10 minutes, 3% H₂O₂ in methanol for 30 minutes, and formic acid for 3 minutes. Sections were again treated with TBS and TTBS for 5 minutes each. The Mouse-to-Mouse detection system kit (Chemicon International, Temecula, CA) containing a pre- and post-antibody blocking solution, Poly horseradish peroxidase goat anti-mouse/rabbit IgG secondary antibody, and 3,3 diaminobenzidine (DAB) substrate was chosen in order to minimize interactions between the monoclonal primary antibody and non-specific mouse tissue epitopes. Sections were incubated with pre-antibody blocking solution for 60 minutes, rinsed in TTBS twice for 5 minutes and incubated with the primary antibody, 4G8 diluted 1:100 in TTBS overnight at 4°C in a humidity chamber. The next morning sections were rinsed twice in TTBS for 10 minutes each, treated with the post-antibody blocking solution for 10 minutes, followed by two more 5 minute TTBS rinses and application of the secondary antibody for 10 minutes followed by two TBS 5 minute rinses. Last, the DAB solution was applied for 2-5 minutes followed by several 5 minute ddH₂O rinses. The sections were air dried, dehydrated (70%, 95%, 100% ethanol and xylene x 2 for each for

5 minutes), coverslipped with Cytoseal 60 (Stephens Scientific, Kalamazoo, MI) and visualized with a light microscope. Brain sections obtained from autopsies performed on AD patients were included as a positive control for both histochemistry methods. Human brain tissues were obtained from postmortem examination at the University of Kentucky Alzheimer's Disease Research Center for the purposes of this laboratory's ongoing research. Tissues were obtained without identifiers and in accordance with all IRB regulations.

The thioflavin-S staining procedure was adapted from the method of Schwartz (1972). Slides were dried and fixed in 4% paraformaldehyde for 10 minutes and fully rinsed with water followed by an incubation in ddH₂O for 5 minutes. Next, sections were stained with 1% aqueous thioflavin-S (ICN Biomedicals, Inc., Aurora, OH) in 50% ethanol for 4-10 minutes and placed in 80% ethanol for two 2 minute incubations followed by two 5 minute ddH₂O washes. The sections finally were dehydrated and coverslipped. Images were visualized by fluorescence microscopy utilizing a fluorescein filter.

Statistical analysis

The wide, continuous distribution in the age of mice used in this study prevented an analysis comparing specific age groups. Therefore, a correlation analysis was employed to determine significant relationships between cholinergic receptor expression (measured by amount of ligand binding in nCi/mg wet tissue) and days of age in both mouse strains. Correlations were performed by Pearson correlation analysis for normally distributed variables. Due to the large number of regions analyzed, data are presented at a significance level of a more conservative $\alpha=0.01$, as well as $\alpha=0.05$. For α -[¹²⁵I]-BTX binding, a post hoc, unpaired, two-tailed Student's t-test was performed to test for differences in genotype means (expressed as nCi/mg wet tissue) ignoring age for the hippocampal regions CA1, CA2, CA3 and posterior hippocampal layer CA3, as well as the zona incerta. All statistical procedures were conducted using GraphPad Prism 4 (San Diego, CA).

Results

Regional changes in α -[¹²⁵I]-BTX binding in APPswe and wild-type mice with age were evaluated and can be found in Table 2.1. Pearson correlation coefficients are presented for APPswe and wild-type mice for each of the brain regions investigated. The effects of aging on α 7 nAChR changes in this mouse model varied significantly between the brain regions analyzed. α 7 nAChR increased in some areas while decreasing in others and no change was seen in additional regions. In detail, as APPswe mice aged, BTX binding increased in several brain regions including the bed nucleus of the stria terminalis and the medial preoptic area at $p < 0.01$. Increased BTX binding in the somatosensory cortex layers 1-3, superior colliculus, substantia nigra, and medial interpeduncular nucleus demonstrated significance at $p < 0.05$. In other brain regions, lateral caudate putamen, lateral hypothalamus, and red nucleus, aging was associated with a significant reduction in BTX binding in both transgenic and wild-type animals. However, decreases in the lateral caudate putamen of wild-type mice and in the red nucleus of APPswe mice showed the strongest correlations ($p < 0.01$). BTX binding decreased with age in wild-type mice in the caudate putamen, the medial subregion of the caudate putamen ($p < 0.01$), and in the inferior colliculus, lateral lemniscus, and dorsal tegmental nucleus to a lesser extent ($p < 0.05$). Among both strains, no changes in BTX binding were seen in the olfactory tubercle, some subregions of the cortex, basal ganglia, and diencephalon. Surprisingly, no changes were detected in either mouse strain in the hippocampus or amygdala with increasing age. Binding similar to background was found in the corpus callosum, a white matter structure lacking nAChR expression, which served as a negative control.

The absence of changes in hippocampal BTX binding with age prompted additional analysis due to the significant role of the hippocampus in learning and memory and its involvement in the pathology of AD. Because no age related changes were found, a post hoc Student's t-test was performed to test for differences in genotype means ignoring age for the hippocampal regions CA1, CA2, CA3 and posterior hippocampal layer CA3, as well as the zona incerta. APPswe mice exhibited an increase in α 7 nAChR binding (nCi/mg wet tissue) compared to wild-type mice in the CA1 ($t[46] = 2.68, p = 0.0101$), CA2 ($t[46] = 2.96, p = 0.0048$), CA3 of the posterior hippocampus ($t[46] = 3.35,$

$p = 0.0016$), and zona incerta ($t[45] = 2.16, p = 0.0359$) for all ages but not the CA3 subfield at the anterior level of the hippocampus (Table 2.1 and Figure 2.1).

The [125 I]-EPI binding assay results for APP^{swe} and wild-type mice during aging are shown in Table 2.2. Decreases in non- $\alpha 7$ nAChR binding occurred in aged animals of both strains in the dorsolateral geniculate nucleus ($p < 0.05$) and superior colliculus ($p < 0.01$). A reduction in binding in the ventrolateral geniculate occurred only in wild-type mice ($p < 0.01$). No significant changes in non- $\alpha 7$ receptors were detected in the olfactory tubercle, neocortex, basal ganglia, or hippocampus of either strain. Mouse genotype means for each region are also listed to illustrate the relative magnitude of binding density.

Table 2.3 presents the results of the [3 H]-QNB binding assay in APP^{swe} and wild-type mice. Wild-type mice demonstrated an increase in total mAChR binding in aging in multiple brain regions including the somatosensory cortex layers 1-3, olfactory cortex, and caudate putamen all significant at $p < 0.05$ and the nucleus accumbens and hippocampus subfield CA1 at a significance level of $p < 0.01$. However, mAChR binding was not affected in APP^{swe} mice in any of the brain regions tested including the neocortex, basal ganglia, and hippocampus. Figure 2.2 depicts representative autoradiographs to demonstrate observed alterations in $\alpha 7$ and non- $\alpha 7$ nAChR and mAChR in APP^{swe} and wild-type mice during aging.

APP^{swe} and wild-type mice from ages 1.5 to 18 months were evaluated for the presence of A β deposition. Both immunohistochemical localization with the antibody, 4G8, and thioflavin-S staining revealed no deposition of the A β peptide at any of the ages tested (Figure 2.3). Sections obtained from clinically documented AD patients were run in parallel to the mouse sections. A high incidence of β -amyloid immunoreactivity and staining was observed on the former sections demonstrating that the failure to detect A β in mouse sections was not due to a technical problem with the assays.

Discussion

The aim of the current study was to investigate the effects of aging on nicotinic cholinergic expression in a transgenic mouse model of Alzheimer's disease. This study demonstrated that during aging, nAChR regulation differs in mice that overexpress APP

compared to the wild-type strain indicative of normal aging. Such cholinergic receptor changes noted in wild-type mice and not in APP^{swe} mice suggest a non-amyloid based mechanism. BTX and EPI bindings showed nAChRs decreased with aging in several brain regions of wild-type mice.

Aging alone has been associated with changes in nicotinic receptors. For example, in the human cortex, studies showed a loss of $\alpha 4\beta 2$ and $\alpha 7$ nAChRs along with $\alpha 4$, $\beta 2$, and $\alpha 7$ mRNA according to a detailed review (Perry et al., 2001). In addition, $\beta 2$ -containing nAChRs were found to be inversely correlated with age in various brain regions of healthy human subjects in a single photon emission computed tomography (SPECT) study (Mitsis et al., 2008). Results from other aging studies in animals vary, and the differences in rodent strains therein makes for a difficult comparison. No change in BTX or EPI binding has been found in aged rats (Tribollet et al., 2004). However, in two common mouse strains tested, the effects of aging on nAChR protein expression measured in the dorsal hippocampus was variable, and expression of the $\alpha 4$ protein was downregulated compared to younger adults in both strains but elevated in astrocytes in one strain. The $\alpha 7$ nAChR was elevated with aging in one strain and the $\beta 4$ subunit was diminished in one of the strains tested (Gahring et al., 2005).

It is possible that presynaptic nAChRs are diminished due to synaptic loss during normal aging. In addition, corticosterone levels could be elevated due to normal aging in the current model which could result in downregulation of nAChRs. Decreases in nicotinic receptor density have been shown to be mediated by exogenous corticosteroids (Pauly and Collins, 1993; Robinson et al., 1996). Therefore, it would have been optimal to measure glucocorticoids in this study to help decipher these changes. Investigating possible alterations in mRNA expression would have also added to the analysis as well.

Muscarinic receptors were examined using QNB binding mainly as a control and by which a means to compare nicotinic receptor binding results. QNB binding revealed a significant increase in total mAChR binding in several regions tested in the wild-type mice. A summary of the three binding results in wild-type and APP^{swe} mice showing the number of brain regions analyzed and the percentage of regions demonstrating a significant correlation with aging (Pearson correlation, $p < 0.05$ and $p < 0.01$) can be found in Table 2.4. The QNB assay displayed an upregulation in 89 percent of the brain

regions tested in the wild-type group, while the BTX and EPI binding assays were significant only in a small minority of regions.

Other reports of muscarinic receptor changes during aging vary among rodent models. In aged Long-Evans rats, there was a significant increase in M2 mAChR receptor binding in the cortex and dentate gyrus of aged Long-Evans rats deemed cognitively impaired by Morris water maze functioning compared to unimpaired rats as well as in the cortex of old compared to young rats, but no change was found in M1 mAChR (Aubert et al., 1995). In contrast, in the same rat strain of similar ages, a decrease in M2 binding was detected compared to young rats in the brainstem and the basal forebrain, which correlated with a Morris water maze spatial learning deficit (Gill and Gallagher, 1998). Interestingly, hippocampal levels of A β 40 were significantly higher in both cognitively impaired and unimpaired aged rats compared to young rats (Vaucher et al., 2001). In autoradiography studies of mAChR receptor density in Fisher 344 rat hippocampus, changes were subregion selective. The M1 and M2 subtype densities were reduced in some layers of areas CA1 and CA3 in adult and aged rats compared to young rats, while in aging M5 was reduced, and M3 and M4 subtypes were enhanced in some hippocampal fields (Tayebati et al., 2002). In the current study, loss of synaptic function occurring during aging may result in a compensatory muscarinic upregulation postsynaptically.

The results herein show a significant increase in BTX binding in various regions in the APP^{swe} mice with age compared to wild-type mice indicating a possible amyloid based mechanism. Further, when examining genotype only, BTX binding was significantly elevated in hippocampal regions in APP^{swe} mice. BTX binding was elevated by approximately 17 percent in hippocampal layer CA1 and was increased by 13 and 10 percent in the CA2 and posterior CA3, respectively. These findings are consistent with other reports that demonstrate elevated α 7 nAChR binding in transgenic mice that overexpress A β , such as the Tg2576 model, and may be due to agonist properties by A β at the α 7 nAChR.

Previous studies have shown that chronic nicotinic agonist treatment increases the expression of nAChRs in rodents (Marks et al., 1983; Schwartz and Kellar, 1983) and smoking upregulates nAChRs in the human brain (Benwell et al., 1988; Nyback et al.,

1989; Wonnacott, 1990; Breese et al., 1997a). The pharmacological interaction between A β and the α 7 nAChR has been well documented. In the brains of AD patients, A β 42 and the α 7 nAChR, but not the α 4 or other subunits, were co-localized in neuritic plaques as well as in cortical and hippocampal neurons in which they interacted with high affinity and form a stable complex (Wang et al., 2000b). Furthermore, A β was shown to bind to the α 7 nAChR with picomolar affinity (Wang et al., 2000b; Wang et al., 2000a). Additionally, A β also binds to non- α 7 nAChRs (Wang et al., 2000a; Fu and Jhamandas, 2003). Several studies suggest A β elicits agonist-like behavior at the α 7 nAChR *in vitro*. (Dineley et al., 2002a; Dougherty et al., 2003). However, there is *in vitro* evidence that A β may inhibit the activity of α 7 nAChRs and to a greater degree non- α 7 nAChRs (Liu et al., 2001; Pettit et al., 2001; Vaucher et al., 2001; Tozaki et al., 2002; Grassi et al., 2003; Lee and Wang, 2003; Wu et al., 2004; Spencer et al., 2005), and A β may also block the activity of mAChRs (Kelly et al., 1996). Moreover, intracerebroventricular injection of A β 42 resulted in antagonism of α 7 nAChRs, as the peptide blocked a hemodynamic response elicited by brain injection of the selective α 7 agonist, choline, confirming A β 's actions *in vivo* (Li and Buccafusco, 2004). It is possible that the contradictory properties of A β at nAChRs may be due to differences in the tissue models, experimental conditions or detection methods employed in these studies. Notably, in a recent report a unique nAChR subtype, α 7 β 2, was discovered in rat basal forebrain cholinergic neurons, and A β demonstrated high affinity binding to this subtype and functional inhibition (Liu et al., 2009). It is likely that lower concentrations of A β in the picomolar to nanomolar range display agonist properties while higher concentrations and further disease progression produces antagonist behavior (Hernandez et al., 2010). Moreover, A β binding to nAChRs would result in upregulation of nAChRs due to agonist or antagonist properties.

Unfortunately, no amyloid deposits were detected in mice tested out to 18 months of age; thus, an evaluation of the effects of A β deposition on nAChRs could not be performed. Amyloid deposition in APP^{swe} mice may have been observed if ages greater than 18 months were tested. Previous reports indicate APP^{swe} mice express a two fold increase in APP compared to wild-type mice, but these reports (Borchelt et al., 1996;

Borchelt et al., 1997; Borchelt et al., 2002) show little or no A β deposition occurring in the age range examined in the current study. However, it has been reported that the APP^{sw} mice used in the current study exhibit elevations in A β 40 and A β 42 concentrations, but only display A β deposition at ages greater than 20 months (Price et al., 1998).

Because the current model is believed to have elevated levels of APP without amyloidosis, it is possible that this mouse model could serve as a paradigm for early AD. The upregulation of α 7 nAChR density in the current study supports the possibility that changes in cholinergic neurotransmission and related cognition occurring early in the course of disease could be due to increased A β concentrations. One would suspect that APP^{sw} mice are exposed to higher soluble A β levels compared with normal aging. However, due to the tissue processing for the autoradiographic methods employed in our study, we were not able to perform investigations of the A β concentrations in these mice to confirm the previous report.

Soluble A β may be a significant mediator in the pathogenesis of AD (Walsh and Selkoe, 2007). Postmortem studies have shown that cortical levels of A β 40 and A β 42 are elevated early in dementia and correlative with the progressive impairment in cognition experienced by AD patients (Naslund et al., 2000). Alzheimer's disease has been described as a disorder of "synaptic failure," and the soluble, oligomeric form of A β , has been used as a predictor of synaptic change (Selkoe, 2002).

In human AD brain, there is a six fold greater concentration of water soluble oligomers of A β compared to controls, most of which are A β 42 and are destined to form insoluble fibrils (Kuo et al., 1996). Intracellular accumulation of A β 42 immunoreactivity occurs before neurofibrillary tangles and A β deposition as an early event especially in the hippocampal and entorhinal cortex pyramidal neurons (Gouras et al., 2000).

Furthermore, neurons that accumulate A β 42 have a high expression of α 7 nAChRs, providing evidence for the selective vulnerability of the cholinergic system in AD (Nagele et al., 2002). Evidence suggests that amyloid plaques form from the cellular lysis of neurons accumulated with A β 42 (D'Andrea et al., 2001). In addition, in human neuroblastoma cells transfected with high levels of α 7, the binding of A β 42 (but not

A β 40) and subsequent intracellular accumulation can be blocked by BTX (Nagele et al., 2002). However, another study found that while the soluble form of A β distinguishes between AD and normal aging, soluble levels of A β 40 and A β 42 are low but most common in normal aging, whereas insoluble levels are much greater in the AD brain. Soluble A β 40 levels were ten fold higher than soluble A β 42/43 levels in AD brains compared to the brains from patients that did not have dementia but showed AD-like pathology. Insoluble A β 40 levels were 20 fold higher in AD patients compared to the non-demented patients with AD-like pathology, and the insoluble A β 42 species was only elevated by two fold (Wang et al., 1999b). One report determined that soluble A β oligomers were found at higher levels in the frontal lobe of AD patients compared to controls and were concentrated at synaptic structures (Kokubo et al., 2005). Levels of soluble A β increased three fold in AD patients and correlated with pathologic hallmarks of disease progression, while insoluble A β was not correlated with disease severity (McLean et al., 1999). Most recently, the progression of cognitive impairment in AD has been correlated with soluble, fibrillar oligomers (Tomic et al., 2009).

The effects of soluble A β have also been demonstrated in mouse models of AD. Mice expressing the APP (V717F) mutation linked to Indiana FAD displayed a loss of neurons, presynaptic nerve terminals, and impairments in synaptic transmission prior to plaque formation. The same group also found that the addition of the APP^{swe} mutation to these mice, leading to elevated levels of A β with a decrease in total APP, led to greater impairments in synaptic transmission in young mice before the presence of plaque formation (Hsia et al., 1999). Total levels of A β 42 in the hippocampus have been shown to correlate with Morris water maze task impairments in 12 month old APP plus PS1 (Borchelt model) mice, when very little deposits were present in the hippocampus (Puolivali et al., 2002). Tg2576 mice show impaired learning and memory at age nine to ten months while increased concentrations of A β 40 and A β 42 were found as early as two months of age, but the presence of amyloid plaques was not assessed until mice reached 12 months of age (Hsiao et al., 1996). Certain aspects of cognitive testing showed gender related impairments in three and nine month old Tg2576 mice well before amyloid deposition (King et al., 1999). Memory impairment assessed by Morris water maze was

found in an AD model expressing wild-type human APP that only forms diffuse deposits but no plaques (Koistinaho et al., 2001). Further, a 56-kDa A β soluble oligomer was found to accumulate extracellularly in the brains of middle aged Tg2576 mice. These middle aged mice 6-14 months old displayed no neuronal dysfunction or plaque accumulation while demonstrating memory impairments in a modified Morris water maze task (Lesne et al., 2006). Finally, through studies of α 7 nAChR genetic deletion in Tg2576 mice, Hernandez and colleagues have proposed that soluble, oligomeric A β activation of α 7 nAChRs results in neuroprotection in early AD through preservation of cholinergic neurotransmission and sequestration of toxic A β (Hernandez et al., 2010).

In contrast, a downregulation in BTX binding occurred in some brain regions in APP^{swe} mice with age. Thus, the relative changes in binding are region specific. This is inconsistent with the possible agonistic properties of A β suggesting some brain regions may only be regulated by normal aging and the accompanying synaptic loss. Due to the predominant presynaptic location of α 7 nAChRs and widespread distribution throughout the CNS, it is also possible A β may affect this receptor differently depending on brain regions and local concentrations. Downregulation may result from increased corticosteroids; however, it is unknown if corticosterone levels in these mice are elevated due to APP expression or normal aging. In Tg2576 mice, abnormal elevations in glucocorticoids occur following restraint stress (Pedersen et al., 1999). Thus, measuring plasma corticosterone levels in this study would provide further insight.

Similarly, reductions were seen in EPI binding in APP^{swe} mice. For example, the reduction of EPI binding in the superior colliculus in APP^{swe} mice with age may suggest a deficit occurs as an early event in AD possibly due to synaptic loss. Interestingly, a downregulation in EPI binding was recently reported in patients with MCI and AD through PET indicating a possible loss early in the disease (Sabri et al., 2008). While other studies have shown changes in α 4 β 2 nAChRs in rodent models and in late stage disease in AD patients, the current results reinforce the selective vulnerability of α 7 nAChRs in AD models demonstrated repeatedly in the literature. The data herein suggest the α 7 nAChR is a sensitive target for regulation by soluble A β in mice that overexpress APP. No changes were found in muscarinic receptor density, as

measured by QNB, in APP^{swe} mice. In contrast to the upregulation seen in BTX binding, it is possible that mAChRs are not upregulated because soluble A β mediated increases in $\alpha 7$ nAChR may stabilize synaptic function and preclude any compensatory changes in muscarinic receptors.

The initial objective of this study was to evaluate changes in $\alpha 7$ nAChR at various ages in the APP^{swe} transgenic mouse model of AD in order to investigate the effects of A β deposition on nicotinic receptor expression. The lack of A β deposition in this model was an obstacle that prevented the characterization of nAChR changes at various stages in the pathogenesis of AD. Furthermore, measuring nAChR mRNA, A β levels, corticosterone, or additional biomarkers would have improved the study.

In conclusion, this study has shown that cholinergic receptor expression was affected by age in APP^{swe} and wild-type mice. Changes in various brain regions suggest a differential regulation of receptor subtypes both by A β and the aging process. While $\alpha 7$ nAChR binding was enhanced in particular brain regions and diminished in others during the aging process, several hippocampal regions, as well as the subthalamic zona incerta, displayed an upregulation in $\alpha 7$ nAChR regardless of age in APP^{swe} mice compared to wild-type controls. This indicates an association with elevated APP expression. While soluble A β is expected to be augmented in the APP^{swe} mice, amyloidosis was not present; this may explain regional differences in cholinergic alterations and nicotinic receptor effects that are of a lesser magnitude than previous studies. The results of this study suggest the $\alpha 7$ nAChR is a sensitive target for regulation by aging and genotype in mice engineered to overexpress the amyloid precursor protein, and changes in $\alpha 7$ nAChR appear prior to amyloid pathology. The mechanism by which APP overexpression affects $\alpha 7$ nAChR binding is yet to be fully determined.

Table 2.1. Modulation of the $\alpha 7$ nAChR, measured by α -[¹²⁵I]-bungarotoxin (BTX) binding, with aging in APPswe and wild-type mice. Data presented are the Pearson correlation coefficient (p-value) for mice 45-558 days of age. The mean \pm standard deviation amount of binding (nCi/mg wet tissue) is also included to illustrate the relative magnitudes for each strain.

Brain Region	Pearson Coefficient (p-value)		Strain Effect	Mean \pm SD	
	Wild-type	APPswe		Wild-type	APPswe
Olfactory cortex					
Olfactory tubercle	-0.191 (0.382)	-0.044 (0.846)		6.98 \pm 1.94	6.72 \pm 1.80
Neocortex					
Somatosensory cortex layers 1-3	0.156 (0.467)	0.456 (0.025)*	\uparrow APPswe	2.63 \pm 0.55	2.60 \pm 0.58
Somatosensory cortex layers 4-6	0.062 (0.772)	0.228 (0.285)		7.86 \pm 1.30	8.11 \pm 1.28
2° somatosensory/insular cortex	-0.148 (0.490)	0.197 (0.355)		2.37 \pm 0.53	2.33 \pm 0.51
Corpus callosum	-0.092 (0.669)	-0.209 (0.327)		0.21 \pm 0.04	0.20 \pm 0.05
Basal ganglia					
Diagonal band	-0.187 (0.381)	-0.025 (0.909)		2.22 \pm 0.53	2.18 \pm 0.45
Caudate putamen (lateral)	-0.556 (0.005)**	-0.488 (0.016)*	\downarrow Both	10.92 \pm 2.08	11.07 \pm 1.71
Caudate putamen (medial)	-0.545 (0.006)**	-0.376 (0.071)	\downarrow Wild-type	4.05 \pm 0.96	4.19 \pm 0.89
Caudate putamen	-0.588 (0.003)**	-0.398 (0.054)	\downarrow Wild-type	5.09 \pm 1.11	5.18 \pm 1.02
Ventral endopiriform	-0.083 (0.702)	-0.326 (0.120)		3.95 \pm 0.80	4.35 \pm 0.77
Dorsal endopiriform	-0.399 (0.054)	-0.359 (0.085)		5.37 \pm 1.38	5.46 \pm 0.89
Bed nucleus (stria terminalis)	0.203 (0.343)	0.601 (0.002)**	\uparrow APPswe	4.58 \pm 0.76	4.46 \pm 0.87
Diencephalon					
Medial preoptic area	0.080 (0.710)	0.532 (0.009)**	\uparrow APPswe	11.60 \pm 2.46	12.04 \pm 2.40
AV thalamic nucleus	-0.361 (0.084)	0.220 (0.313)		8.95 \pm 2.07	8.84 \pm 1.56
Lateral hypothalamus (anterior)	0.046 (0.833)	0.331 (0.123)		11.41 \pm 1.59	12.15 \pm 1.19
Lateral hypothalamus (posterior)	-0.478 (0.018)*	-0.524 (0.010)*	\downarrow Both	12.89 \pm 2.22	13.03 \pm 1.70
Ventrolateral geniculate nucleus	-0.152 (0.478)	0.021 (0.925)		13.01 \pm 1.74	13.46 \pm 1.25
Premammillary nucleus	-0.056 (0.795)	-0.057 (0.791)		12.97 \pm 2.47	13.74 \pm 2.87
Subthalamic nucleus	-0.142 (0.508)	-0.254 (0.243)		16.14 \pm 1.96	16.59 \pm 1.40
Zona incerta	-0.175 (0.414)	-0.294 (0.173)		2.86 \pm 0.53	3.17 \pm 0.42
Parafascicularis nucleus	-0.160 (0.454)	-0.071 (0.747)		24.40 \pm 2.84	24.68 \pm 2.39
Posterior hypothalamic area	0.066 (0.764)	0.340 (0.113)		11.91 \pm 1.46	12.00 \pm 1.03

Continued

Table 2.1 (Continued)

Brain Region	Pearson Coefficient (p-value)		Strain Effect	Mean ± SD	
	Wild-type	APPswe		Wild-type	APPswe
Anterior hippocampus					
CA1 layer	0.141 (0.512)	0.070 (0.744)		3.82 ± 0.69	4.48 ± 0.99
CA2 layer	-0.311 (0.140)	-0.089 (0.679)		11.71 ± 1.96	13.18 ± 1.45
CA3 layer	-0.073 (0.733)	-0.093 (0.666)		5.59 ± 0.97	5.91 ± 0.83
Posterior hippocampus					
CA3 layer	0.039 (0.856)	-0.234 (0.271)		15.60 ± 1.48	17.12 ± 1.68
Dentate gyrus (molecular blade)	-0.026 (0.903)	0.029 (0.894)		4.40 ± 1.13	4.97 ± 0.91
Dentate gyrus (hilar blade)	0.222 (0.296)	0.275 (0.194)		26.62 ± 2.90	26.41 ± 2.08
Amygdala					
Medial amygdala	-0.149 (0.488)	-0.227 (0.286)		26.82 ± 1.90	26.38 ± 2.83
Mesencephalon					
Superior colliculus	0.106 (0.622)	0.472 (0.020)*	↑APPswe	21.42 ± 1.72	21.58 ± 1.50
Substantia nigra	0.325 (0.130)	0.496 (0.019)*	↑APPswe	6.67 ± 1.09	6.79 ± 1.11
Interpeduncular nucleus (medial)	-0.032 (0.882)	0.465 (0.022)*	↑APPswe	16.36 ± 2.59	17.61 ± 2.13
Interpeduncular nucleus (lateral)	0.218 (0.306)	0.360 (0.084)		19.83 ± 3.18	20.51 ± 2.51
Interpeduncular nucleus (central)	-0.264 (0.224)	0.205 (0.337)		0.96 ± 0.24	1.13 ± 0.41
Red nucleus	-0.422 (0.040)*	-0.605 (0.002)**	↓Both	24.80 ± 2.73	24.11 ± 2.63
Inferior colliculus	-0.460 (0.027)*	-0.193 (0.365)	↓Wild-type	29.26 ± 2.62	29.18 ± 1.65
Pons					
Lateral lemniscus	-0.498 (0.013)*	-0.182 (0.396)	↓Wild-type	18.13 ± 1.73	18.21 ± 2.71
Dorsal tegmental nucleus	-0.413 (0.045)*	0.027 (0.900)	↓Wild-type	34.37 ± 1.86	33.92 ± 4.12

* denotes significance of $p < 0.05$

** denote significance of $p < 0.01$

Arrows designate direction of significant strain effect

Table 2.2. The effects of aging in APPswe and wild-type mice on non- $\alpha 7$ nAChR expression, measured by [125 I]-epibatidine (EPI) binding. Data presented are the Pearson correlation coefficient (p-value) for mice 45-558 days of age. The mean \pm standard deviation amount of binding (nCi/mg wet tissue) is also included to illustrate the relative magnitudes for each strain.

Brain Region	Pearson Coefficient (p-value)		Strain Effect	Mean \pm SD	
	Wild-type	APPswe		Wild-type	APPswe
Olfactory cortex					
Olfactory tubercle	-0.249 (0.240)	-0.165 (0.464)		3.9 \pm 0.7	4.1 \pm 0.8
Neocortex					
Somatosensory cortex layers 1-3	-0.320 (0.127)	0.014 (0.949)		3.8 \pm 0.7	3.9 \pm 0.6
Somatosensory cortex layers 4-6	-0.043 (0.843)	-0.150 (0.494)		2.4 \pm 0.1	2.4 \pm 0.1
Basal ganglia					
Caudate putamen	-0.337 (0.107)	0.019 (0.932)		5.2 \pm 0.9	5.6 \pm 1.0
Diencephalon					
Anterior thalamus	-0.391 (0.065)	-0.134 (0.551)		20.5 \pm 1.9	20.6 \pm 2.3
Dorsolateral geniculate nucleus	-0.479 (0.018)*	-0.454 (0.034)*	↓Both	20.7 \pm 1.8	21.0 \pm 2.2
Ventrolateral geniculate nucleus	-0.578 (0.003)**	-0.338 (0.124)	↓Wild-type	19.6 \pm 1.8	20.1 \pm 2.4
Zona incerta	-0.197 (0.356)	-0.321 (0.145)		6.2 \pm 1.2	6.5 \pm 1.4
Hippocampus					
Dentate gyrus (lateral blade)	-0.115 (0.592)	-0.132 (0.538)		3.5 \pm 0.3	3.6 \pm 0.3
Dentate gyrus (medial blade)	-0.220 (0.302)	-0.218 (0.305)		3.4 \pm 0.4	3.5 \pm 0.3
Subiculum	-0.055 (0.799)	-0.080 (0.715)		9.2 \pm 1.3	9.5 \pm 1.8
Mesencephalon					
Superior colliculus	-0.701 (0.0002)**	-0.602 (0.002)**	↓Both	21.6 \pm 1.8	21.4 \pm 2.4

* denotes significance of $p < 0.05$

** denote significance of $p < 0.01$

Arrows designate direction of significant strain effect

Table 2.3. The effects of aging in APPswe and wild-type mice on mAChR expression, measured by [³H]-quinuclidinyl benzilate (QNB) binding. Data presented are the Pearson correlation coefficient (p-value) for mice 45-558 days of age. The mean ± standard deviation amount of binding (nCi/mg wet tissue) is also included to illustrate the relative magnitudes for each strain.

Brain Region	Pearson Coefficient (p-value)		Strain Effect	Mean ± SD	
	Wild-type	APPswe		Wild-type	APPswe
Olfactory cortex					
Olfactory tubercle	0.450 (0.046)*	0.358 (0.122)	↑Wild-type	6.9 ± 0.9	6.7 ± 1.1
Neocortex					
Somatosensory cortex layers 1-3	0.516 (0.017)*	0.0312 (0.893)	↑Wild-type	5.4 ± 0.7	5.2 ± 0.7
Somatosensory cortex layers 4-6	0.387 (0.083)	-0.262 (0.252)		3.6 ± 0.4	3.5 ± 0.5
Basal ganglia					
Caudate putamen	0.437 (0.047)*	-0.003 (0.990)	↑Wild-type	5.2 ± 0.8	5.1 ± 0.9
Nucleus accumbens	0.676 (0.001)**	0.216 (0.361)	↑Wild-type	6.4 ± 1.1	6.0 ± 1.0
Hippocampus					
Layer CA1	0.543 (0.009)**	0.060 (0.796)	↑Wild-type	6.6 ± 1.1	6.3 ± 1.5
Layers CA2 and CA3	0.455 (0.033)*	-0.002 (0.993)	↑Wild-type	4.3 ± 0.5	4.1 ± 0.7
Dentate gyrus (lateral blade)	0.454 (0.034)*	-0.022 (0.925)	↑Wild-type	6.0 ± 0.9	5.6 ± 1.4
Dentate gyrus (medial blade)	0.414 (0.056)*	-0.062 (0.789)	↑Wild-type	6.9 ± 0.8	6.4 ± 1.2

* denotes significance of $p < 0.05$

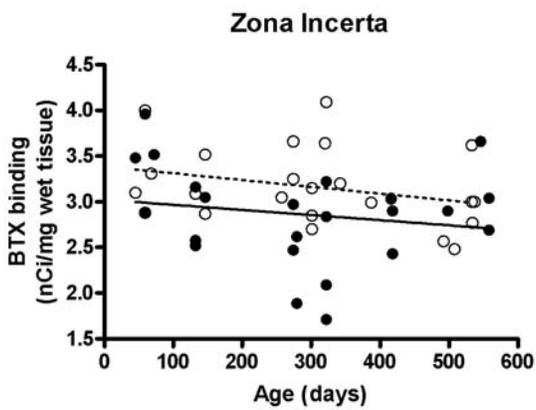
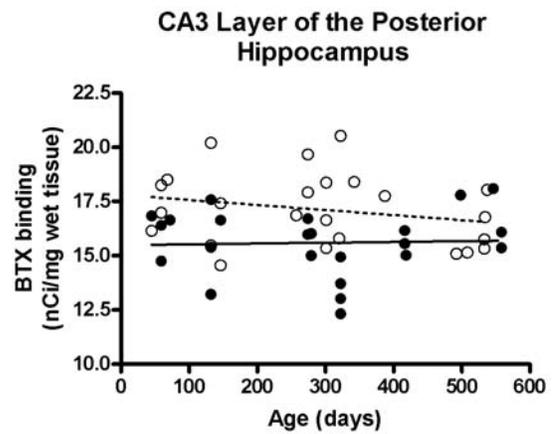
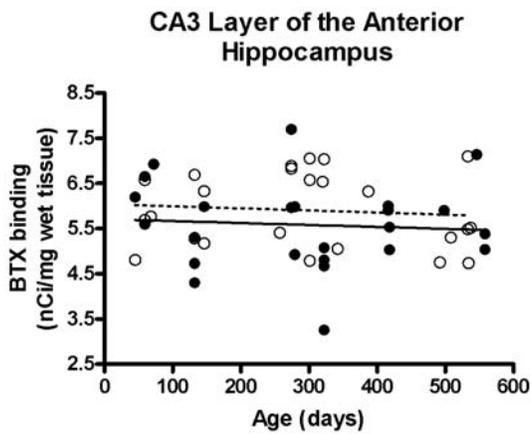
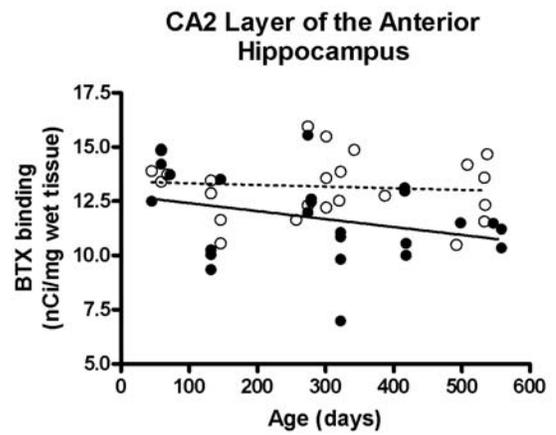
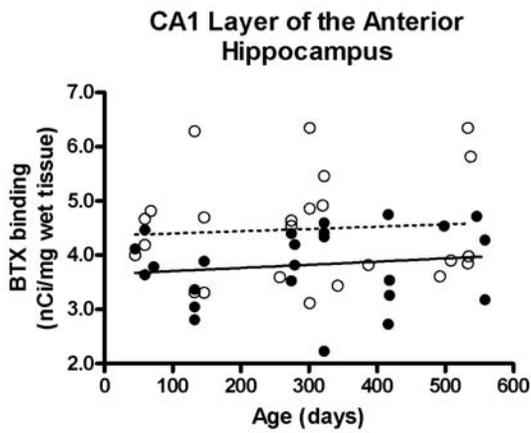
** denote significance of $p < 0.01$

Arrows designate direction of significant strain effect

Table 2.4. A summary of the binding results in APPswe and wild-type mice shows the number of brain regions analyzed for each of three binding studies and the number of regions displaying a significant change with aging determined by Pearson correlation analysis ($p < 0.05$ and $p < 0.01$).

Binding Study	Regions	Wild-type		APPswe	
		Increase	Decrease	Increase	Decrease
BTX	38	0	8 (21%)	6 (16%)	3 (8%)
EPI	12	0	3 (25%)	0	2 (17%)
QNB	9	8 (89%)	0	0	0

Abbreviations: BTX, α -[¹²⁵I]-bungarotoxin; EPI, [¹²⁵I]-epibatidine; QNB, [³H]-quinuclidinyl benzilate



- Wild-type
- APPsw
- Wild-type
- - - APPsw

Figure 2.1. Enhanced $\alpha 7$ nAChR expression in APPswe mice compared to wild-type controls. Correlation analysis demonstrated no difference in BTX binding as a function of age in the hippocampus of both mouse strains; however, a difference between APPswe and wild-type strains was apparent without considering age. Therefore, a post hoc Student's t-test was performed to test for differences in genotype means disregarding age for the hippocampal regions CA1, CA2, CA3 and posterior hippocampal layer CA3, as well as the zona incerta. APPswe mice exhibited an increase in $\alpha 7$ nAChR binding compared to wild-type mice in the CA1 ($t[46] = 2.68, p = 0.0101$), CA2 ($t[46] = 2.96, p = 0.0048$), CA3 of the posterior hippocampus ($t[46] = 3.35, p = 0.0016$), and zona incerta ($t[45] = 2.16, p = 0.0359$) for all ages but not the anterior subfield CA3 of the anterior hippocampus.

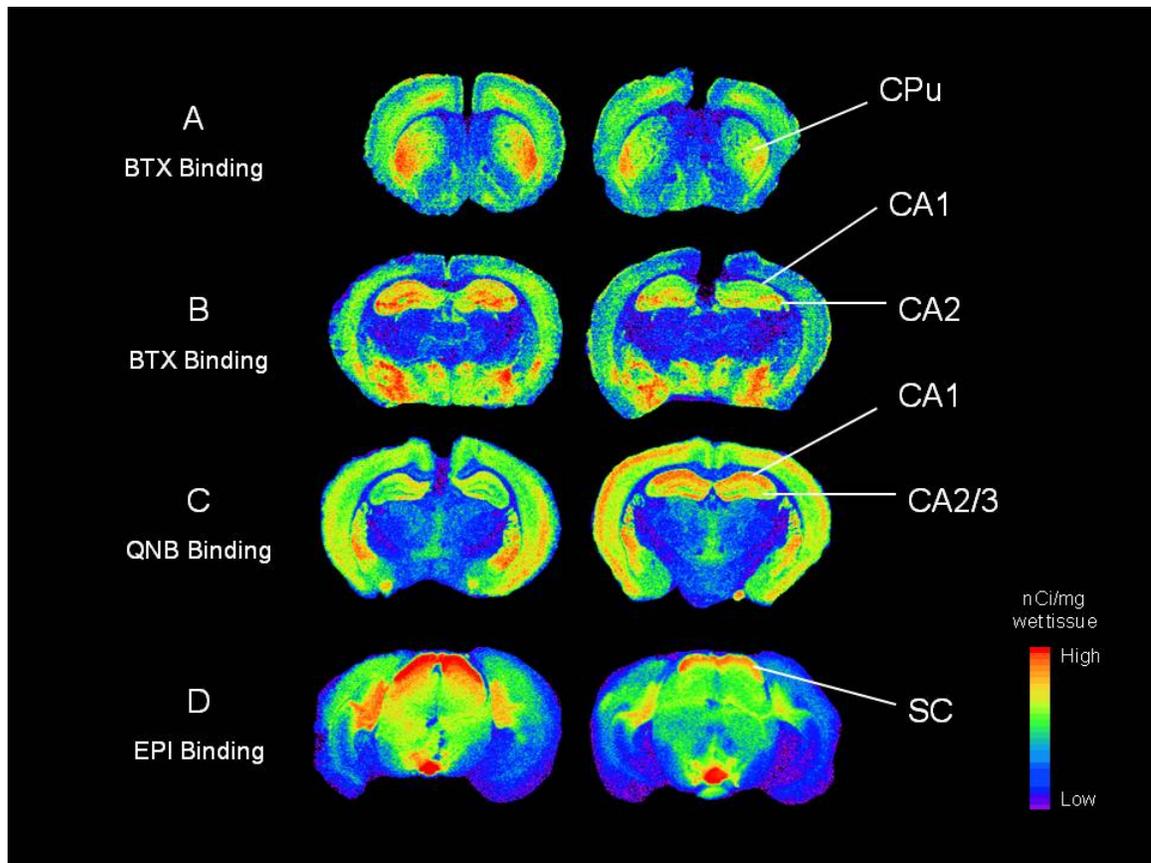


Figure 2.2. Representative autoradiographs demonstrate cholinergic receptor binding in APPswe and wild-type mice in the aging study. Panel A shows the significant decrease in $\alpha 7$ nAChR binding in the caudate putamen in young wild-type (left) compared to aged wild-type (right) mice. In Panel B, a significant increase in $\alpha 7$ nAChR binding in hippocampal CA1 and CA2 is demonstrated in APPswe (left) compared to wild-type (right) mice regardless of age ($p < 0.05$, Student's t-test). Panel C shows the significant increase in total mAChR binding in the hippocampus of young wild-type (left) compared to aged wild-type (right) mice. Panel D displays the significant decrease in non- $\alpha 7$ nAChR binding in the superior colliculus in young APPswe (left) compared to aged APPswe (right) mice. Abbreviations: CPu: caudate putamen; SC: superior colliculus

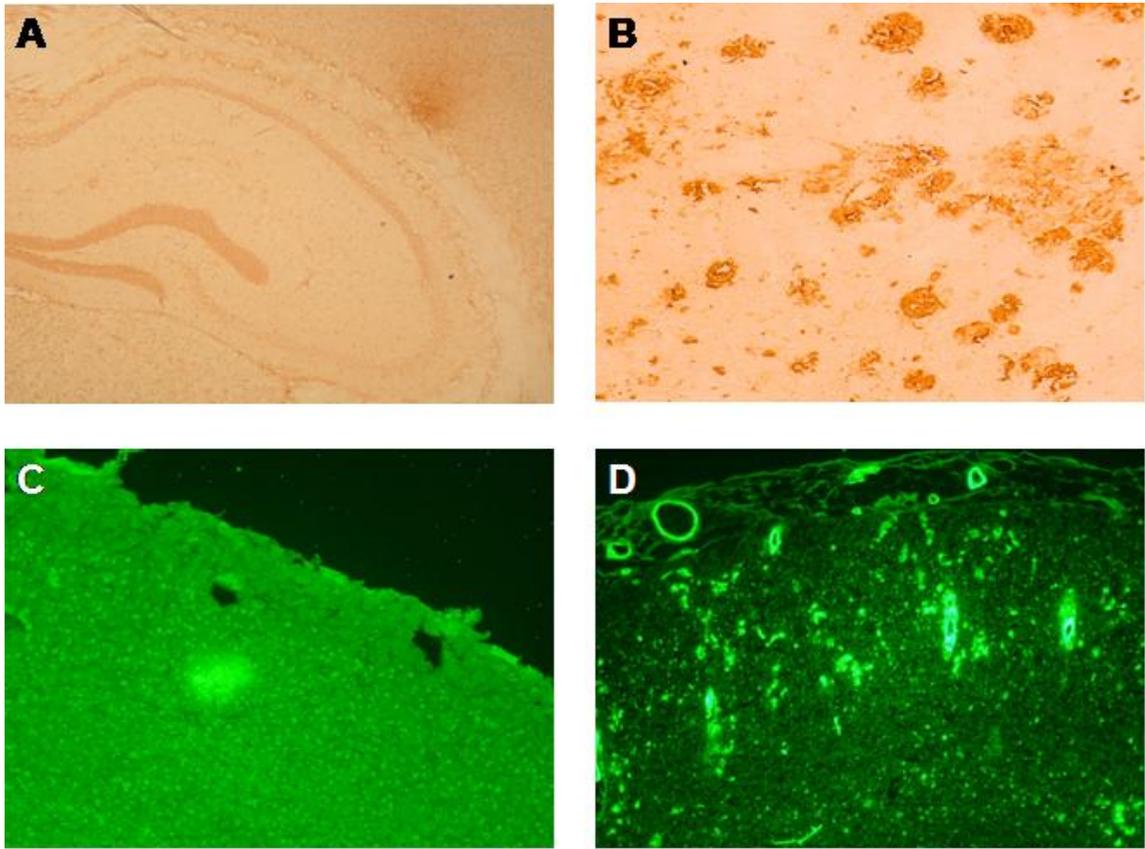


Figure 2.3. β -amyloid histochemistry was employed for the detection of $A\beta$ deposition in APPswe mice. Both immunohistochemistry and staining techniques revealed no $A\beta$ peptide deposition in the APPswe mice used in the current study. The top panels represent immunohistochemical localization with the monoclonal antibody, 4G8. An 18 month old APPswe mouse is depicted in panel A. For comparison, panel B shows $A\beta$ containing neuritic plaques in postmortem brain tissue from a patient with Alzheimer's disease. The bottom panels show thioflavin-S staining for $A\beta$ in an aged APPswe mouse (C) and a human Alzheimer brain (D).

Chapter 3: Galantamine Treatment Following Traumatic Brain Injury in Rats: Effects on Cognition and nAChR Expression

Introduction

Each year in the U.S., about 1.4 million individuals suffer from traumatic brain injury (TBI) (Langlois et al., 2006), and approximately 5.3 million Americans are living with long-term functional changes in thinking, sensation, language, and emotion as a result of TBI (Thurman et al., 1999; NINDS, 2002). Characterized by deficits in arousal, attention, memory, and executive functioning, impairments in cognition are among the most persistent and debilitating neuropsychiatric sequelae of TBI (Arciniegas, 2003).

Both clinical and experimental investigations have demonstrated a disruption in cholinergic neurotransmission following TBI. In a clinical study, imaging and neuropsychological examinations linked the cognitive sequelae of TBI to the ACh containing regions of the basal forebrain as evidenced by reduced grey matter densities (Salmond et al., 2005). Attempts have been made to treat the cognitive sequelae of TBI with cholinergic enhancing drugs just as in Alzheimer's disease. Patients with AD or TBI may exhibit similar memory dysfunction and other cognitive impairments. There are several reports of the clinical evaluation, through clinical trials or clinical experience, of AChEIs following TBI, most of which have been conducted with donepezil (Aricept®), tacrine (Cognex®), or physostigmine resulting in cognitive improvement (Levin et al., 1986; Cardenas et al., 1994; Pike et al., 1997; Taverni et al., 1998; Masanic et al., 2001; Zhang et al., 2004; Khateb et al., 2005).

Acetylcholinesterase inhibitors (AChEIs), including galantamine, are a class of drugs used in the treatment of Alzheimer's disease and are the subject of ongoing research. Galantamine hydrobromide, marketed as Razadyne® ER (extended release) and Razadyne®, formerly Reminyl®, is FDA approved to improve cognition and activities of daily living and slow the progression of cognitive symptoms in patients with mild to moderate Alzheimer's disease (Robinson and Plosker, 2006). In one clinical trial in Alzheimer's patients, long term treatment with galantamine resulted in an 18 month period without cognitive decline (Raskind et al., 2004). Likewise, in a study that

followed AChEI use in patients with TBI related chronic impairments including target symptoms such as poor memory, patients treated with long term galantamine for 6-33 months as well as those treated with other AChEIs expressed benefits in functioning (Tenovuo, 2005).

Some studies have shown that the efficacy of AChEIs for cognitive enhancement does not necessarily correlate with the potency of enzyme inhibition. Galantamine is a long acting, centrally active, selective, competitive and reversible AChEI. While galantamine is only a modest AChEI, it acts as an allosteric potentiating ligand (APL) for both $\alpha 7$ and $\alpha 4\beta 2$ nicotinic receptor (nAChR) subtypes. Studies have shown that galantamine may elicit a neuroprotective effect directly through its actions on nAChRs (Coyle et al., 2007; Wang et al., 2007). Other cholinesterase inhibitors such as donepezil and physostigmine have demonstrated cognitive improvement; however, the strong APL activity of galantamine suggests its ability to enhance nicotinic receptor activation may be prominently involved in its neuroprotective effects.

APLs (Schrattenholz et al., 1996; Maelicke et al., 2001), also known as positive allosteric modulators (PAMs) are receptor sensitizing agents that elicit their actions through the interaction on a binding site other than the classical agonist binding pharmacophore. Ligand gated ion channel receptors commonly express multiple binding sites for APLs, one such example is the GABA_A receptor. As a result of APL activity, nAChRs do not undergo compensatory changes such as receptor desensitization or changes in expression or density (Pereira et al., 1993; Pereira et al., 1994; Schrattenholz et al., 1996). The $\alpha 7$ nicotinic acetylcholine receptor (nAChR) is a homomeric ligand gated ion channel prominently located in hippocampal and cortical regions of the rodent brain. The high calcium permeability of the $\alpha 7$ nAChR (Seguela et al., 1993) makes it unique among nicotinic receptor subtypes. Although the endogenous functions of $\alpha 7$ receptors are not clearly understood, previous studies have implicated these proteins in processes including learning and memory, synaptic plasticity, neuroprotection, inflammation and presynaptic regulation of neurotransmitter release (Levin et al., 2002). Clinical conditions that may be related to alterations in $\alpha 7$ nAChR density and/or function include Alzheimer's disease and schizophrenia.

Previous studies from our laboratory have shown that experimental traumatic brain injury causes a widespread and significant loss of $\alpha 7$ nAChR binding in hippocampal and cortical brain regions (Verbois et al., 2000; Verbois et al., 2002). $\alpha 7$ receptor downregulation occurs rapidly following TBI (within one hour), and persists for at least 21 days in some brain regions. Moreover, TBI is associated with cognitive impairment demonstrated in the Morris water maze task of learning and memory, and treatment with nicotine (Verbois et al., 2003b; Verbois et al., 2003a) partially attenuates this deficit.

Our laboratory has previously studied the effects of the pharmacological modulation of nAChRs as a means to improve neurological outcomes following experimental TBI. Nicotine 0.3mg/kg administered intraperitoneally twice daily for 11 days following a mild to moderate 1.5 mm CCI to the somatosensory cortex partially attenuated deficits in the training phase (distance traveled to the platform) but fully attenuated deficits in the retention probe test. In addition, cognitive improvements were accompanied by enhanced non- $\alpha 7$ nAChR binding in the cerebral cortex and striatum, regions which showed reductions following CCI. Upregulation of $\alpha 7$ nAChRs was not demonstrated in the hippocampal and cortical regions in which TBI related deficits were noted (Verbois et al., 2003b). However, when chronic nicotine was continuously administered by osmotic mini-pump for seven days, there was an attenuation of $\alpha 7$ nAChR deficits in several hippocampal regions such as the CA2, CA3, and hilar and lateral blades of the dentate gyrus (Verbois et al., 2003a). No cortical tissue sparing ability of nicotine was apparent when given intermittently; however, continuous infusion of 0.125 or 0.25 mg/kg/hour resulted in a significant sparing effect. In addition, a study of the $\alpha 7$ nAChR selective ligand, choline, was performed in the CCI model of experimental TBI. Choline administered in the diet for two weeks prior to injury and just under two weeks following injury resulting in significant cortical tissue sparing indicative of neuroprotection and some improvement in spatial memory impairments evaluated using the Morris water maze task. In the superior colliculus and the CA1 subfield of the hippocampus, choline attenuated deficits in BTX binding associated with the injury (Guseva et al., 2008).

Hence, our studies have shown deficits in $\alpha 7$ nAChR expression that may contribute to cognitive dysfunction induced by TBI, and pharmacologic modulation of $\alpha 7$ nAChRs may result in some functional improvement. We hypothesized that galantamine, an AChEI and APL for nAChRs, facilitates neuroprotection and cognitive enhancement in an experimental model of traumatic brain injury. The purpose of this study was to evaluate the effects of the nAChR modulator, galantamine, on cognition and nAChR expression in rats following experimental brain injury.

Materials and Methods

Animal surgeries and study design

Rats were housed in a temperature controlled room on a 12 hour light/dark cycle at the University of Kentucky for at least one week prior to surgeries and were allowed unlimited access to food and water. All procedures were done in accordance with the University of Kentucky IACUC guidelines. Adult male Sprague-Dawley rats (mean weight = 308 g) were randomly assigned to receive a mild to moderate 1.5 mm controlled cortical impact (CCI) brain injury or sham craniotomy surgery (Scheff et al., 1997; Scheff and Sullivan, 1999; Scheff et al., 2005). Rats were anaesthetized with 4% isoflurane and immobilized in a Kopf stereotaxic frame. Craniotomy (6 mm) was performed using a Michele trephine (Miltex, Lake Success, NY) midway between bregma and lambda above the somatosensory cortex (bregma -2.8 mm, 2.5 mm lateral), and the skull cap was carefully removed. An electronically controlled nitrogen driven piston (TBI-0300 Impactor, Precision Systems and Instrumentation, LLC) was then placed on the surface of the exposed brain and used to administer a 1.5 mm cortical deformation 5 mm in diameter at a target velocity of 3.5 m/s. Sham animals underwent identical procedures without impaction. After the skull cap was replaced, surgical seal (Johnson & Johnson, Arlington, TX), dental acrylic, and staples were applied, and rats were placed back in their cages.

Study 1. Twenty-four hours following injury, rats were randomized into three treatment groups to receive twice daily intraperitoneal (i.p.) injections of 3.3 mg/kg galantamine (n = 7), 0.3 mg/kg nicotine free base (n = 7) or 0.9% (normal) saline vehicle (n = 7). All drug treatments were continued throughout the duration of the study

including the cognitive evaluation portion, totaling 15 days, and sham operated animals (n = 7) received only saline. Galantamine hydrobromide 3.3 mg/ml (Tocris Bioscience, Ellisville, MO) and nicotine free base 0.3mg/ml were prepared as stock dosing solutions in normal saline, pH 7.4, sterile filtered, aliquoted and stored according to manufacturers' recommendations. Rats were weighed each morning and drugs were administered at 8 am and 6 pm on the treatments days in volumes based on their morning weight. For example, a rat weighing 300 g would receive an i.p. injection of 0.3 ml. On the morning of the eleventh day post-injury, the Morris water maze behavioral task was commenced to assess spatial memory.

Study 2. The same methods as in study 1 were employed in a second study to evaluate the efficacy of various different galantamine exposure regimens. Galantamine 3.3 mg/kg was administered twice daily intraperitoneally (a) 30 minutes post-injury, again that evening, and twice daily on the day after surgery (GAL1-2, n = 6), (b) beginning 24 hours after injury and continuing on days 2-6 following surgery (GAL2-6, n = 6), or (c) on days 7-11 at which time the Morris water maze testing was conducted (GAL7-11, n = 4). Normal saline twice daily was given on days 1-11 (n = 6).

Morris water maze behavioral task

The Morris water maze spatial navigation task evaluating learning and memory is described in detail elsewhere (Morris, 1984) and was employed using specifications listed previously (Scheff et al., 1997; Verbois et al., 2003b). The task consisted of four trials each on five consecutive days in which rats were given 60 seconds to find a hidden escape platform (13.5 cm) in a circular pool of water (127 cm diameter by 56 cm height). Black tempera paint was added to the pool in order to conceal the platform. Each of the four walls displayed spatial cues. The rats were placed in the pool from a different quadrant on each of the four separate trials with a five minute interval between trials. If the rat failed to find the platform, the rat was placed on the platform at the end of the 60 second trial. Three hours after the last trial on day five, the rats were placed in the pool for a 60 second memory retention test in which the hidden platform was removed. Only the first 15 seconds was used in the retention analysis. All tests were video recorded from above and a video motion analyzer (Videomex V, Columbus Instruments,

Columbus, OH) with Videomex-ONE software was used to assess the swim strategy and speed.

Tissue preparation and nissl staining

For both studies, immediately following completion of five days of behavioral testing, animals were euthanatized by decapitation and brains were excised, snap frozen in isopentane, and sectioned 16 microns thick using a Leica CM50 cryostat (Nussloch, Germany). Tissues were then mounted onto slides coated with gelatin, chromium potassium sulfate and poly-L-lysine to promote tissue adherence. Serial sets of adjacent tissue sections were collected throughout the entire rostro-caudal axis of the rat brain, beginning at approximately plate 15 (bregma 0.70 mm) and ending at approximately plate 44 (bregma -6.72 mm) according to Paxinos and Watson's rat brain atlas (Paxinos and Watson, 1986). Serial sets were obtained to evaluate nAChR radioligand binding focusing on cortical and hippocampal brain regions as well as nissl staining for cortical sparing analysis. Sections were stored at -80°C until use at which time they were thawed and air dried. All reagents were purchased from either Sigma-Aldrich (St. Louis, MO) or Fisher-Scientific (Pittsburgh, PA) unless otherwise stated.

Nissl staining of the smooth endoplasmic reticulum, was performed for cortical sparing analysis of the treatments employed. Cresyl violet stain was made by combining 6% aqueous cresyl violet stock with 0.2 M acetic acid, 0.2 M sodium acetate and ddH₂O and was filtered twice. Next, the samples were submerged two minutes in each of the following: 100% ETOH, xylene, 100% ETOH, 95% ETOH, 70 % ETOH, ddh₂o. This was followed by an incubation in the cresyl violet stain for 10 minutes. Next, samples were dipped three times each in 70% ETOH, 95% ETOH, 100% ETOH and then for 30 seconds in 100% ETOH. The process was completed by submerging the samples in Citrisolv Fisherbrand ® (substituted for xylene) for 2 minutes and once again for at least another 2 minutes. Coverslips were then applied with Cytoseal.

Cholinergic receptor autoradiography

Quantitative receptor autoradiography was performed to investigate nicotinic receptor binding using the radioligands, α -[¹²⁵I]-bungarotoxin and [¹²⁵I]-epibatidine, selective for the binding of α 7 nAChRs and non- α 7 nAChRs, respectively. All

radioligands were purchased from PerkinElmer Life Sciences, Inc., Boston, MA. α - ^{125}I -bungarotoxin (BTX) binding was carried out as previously described by Pauly and Collins (1993). The concentration of radioligand used was 2-3 fold greater than the affinity (K_d) for receptor binding to ensure the assay results are representative of changes in receptor number (B_{max}) as opposed to alterations in affinity. In detail, brain sections were first pre-incubated in Krebs-Ringer HEPES (KRH) buffer, pH 7.5 (1180 mM NaCl, 48 mM KCl, 25 mM CaCl_2 , 12 mM $\text{MgSO}_4(7\text{H}_2\text{O})$, 200 mM HEPES, and 100 mM NaOH) for 30 minutes at room temperature. Next, 2.5 nM α - ^{125}I -BTX (specific activity = 154.0 Ci/mmol) was added to KRH buffer containing 0.5 mg/ml bovine serum albumin, to protect the radioligand from proteases and limit adherence to plastic or glass, in which sections were incubated for two hours at room temperature. This was followed by a series of washes (three x 20 minutes in KRH, one x 10 seconds in 10% KRH, and one x 10 seconds in double deionized water [ddH₂O]) at 4°C.

Non- $\alpha 7$ nAChR binding was determined through the use of ^{125}I -epibatidine (EPI), specific activity = 2200 Ci/mmol, according to the method of Perry and Kellar employing tritiated EPI (Perry and Kellar, 1995). ^{125}I -EPI possesses similar properties to the tritiated form such as a high affinity and low non-specific binding (Davila-Garcia et al., 1997; Whiteaker et al., 2000). However, due to a significantly higher specific activity of the iodinated form and limited resources, it was not possible to purchase the radioligand in sufficient concentration to conduct assays at saturation. Therefore, the saturating concentration of 400pM was obtained by adding a sufficient amount of cold EPI (5mg vial, FW 281.6; adding amount of EPI needed to increase hot EPI amount to 400pM). Brain sections were incubated with EPI in a pH 7.0 buffer containing 50 mM Tris HCl, 120 mM NaCl, 5 mM MgCl_2 , and 2.5 mM CaCl_2 for 40 minutes at room temperature. The incubation was followed by a number of washes at 4°C (two x 5 minutes in buffer, one x 10 seconds in 10% buffer, and one x 10 seconds in ddH₂O).

Once the washes were completed, sections were dried under a low speed fan and stored overnight under vacuum in a desiccator at room temperature. The following day, sections from the BTX and EPI binding assays were exposed to Kodak Biomax MR film. Exposures were stored in x-ray cassettes along with slides containing calibrated brain tissue paste standards with known amounts of ^{125}I for six days or three weeks (19 days)

for BTX and EPI binding assays, respectively. Tissue standards were prepared as described elsewhere (Geary et al., 1985; Pauly and Collins, 1993). Films were processed with Kodak GBX developer and Photoflo 200 solution (Eastman Kodak Co., Rochester, NY).

Image analysis of radioligand binding and cortical sparing and statistics

Brain images were illuminated with a Northern Lights desktop illuminator (Model B95 Imaging Research, Ontario, Canada). Binding films and nissl stained sections were analyzed using NIH image v1.59 software on a Power Macintosh connected to a Sony XC-77 CCD camera via a Scion LG-3 frame-grabber or using ImageJ v1.34j software on an iMac employing a Scion CFW-1310M digital camera. For radioligand binding data, a tissue radioactivity versus optical density standard curve, fit to a third degree polynomial, was generated to determine molar quantities of bound ligand, expressed as nCi/mg of wet tissue weight, from measured optical densities of the brain regions of interest. Each brain structure was measured on the ipsilateral and contralateral sides separately in multiple consecutive brain sections. Cortical sparing analysis was conducted as previously described (Scheff and Sullivan, 1999). Eleven (study 1) or nine (study 2) equally spaced nissl stained cryosections through the injured area were used from each animal. A standard office ruler was used to measure the number of pixels contained in a specified millimeter distance. Cortical area was then measured on each side of the brain separately using the corpus callosum and lamina 1 as boundaries. The percentage of cortical tissue spared was calculated by dividing the mean cortical area for the ipsilateral hemisphere by the mean cortical area for the contralateral side of the brain, multiplied by 100.

Statistical analysis was performed using a two-way (treatment, side), repeated measures, analysis of variance (ANOVA) followed by a Tukey-Kramer multiple comparisons test (GBSTAT software) for quantitative autoradiography of nAChRs. A two-way (treatment, day), repeated measures, ANOVA followed by a Tukey-Kramer multiple comparisons test was used to analyze the body weight and the Morris water maze acquisition data. A one-way analysis of variance was used for all other comparisons of the Morris water maze and cortical sparing data (GBSTAT software or

Graphpad Prism 5.01, San Diego, CA). Statistical significance was determined at an alpha level of 0.05.

Results

Study 1. The body mass following TBI in rats treated with galantamine, nicotine or saline is shown in figure 3.1. Rats were first weighed immediately before receiving a 1.5 mm CCI and again each morning before receiving study drugs for the next 15 days. The body mass was obtained in order to determine the volume of drug to be given in each dose. Two-way repeated measures ANOVA demonstrated a significant effect of day, $F(15, 360) = 54.35, p < 0.0001$. In all treatment groups, rats lost weight the day following surgery. Body mass was significantly lower by day 2 in galantamine and saline treated rats, and by day 3 the nicotine treated rats weighed significantly less. Body mass in the sham group during the initial days following injury was not significantly different compared to baseline. After the initial drop in weight on the day following TBI, Galantamine and nicotine treated rats gained weight throughout the study, but their body mass was not statistically different on the final study day compared to the first study day. In comparison, the body mass of the sham group was significantly elevated on day 9 until the end of the study. Likewise, the saline treated rats displayed an elevated body mass beginning on day 13 compared to day 1. Nevertheless, ANOVA revealed no treatment group effect; thus, there was no differences in body mass between groups at the end of the study.

The Morris water maze behavioral task of spatial learning and memory was performed in rats receiving galantamine, nicotine, or saline following CCI. Results of the acquisition phase of testing are presented in Figure 3.2. Panel A shows the training phase of the Morris water maze, in which each rat performed four swim trials on five consecutive days. The path length traveled to find the platform was used for analysis instead of latency to platform goal, as differences in swim speed would affect this parameter. Two-way repeated measures ANOVA for path length revealed a significant effect of treatment group $F(3, 432) = 8.54, p < 0.0001$, day $F(4, 432) = 9.12, p < 0.0001$, and interaction $F(12, 432) = 2.15, p = 0.0135$.

When compared to the first day of training, the Tukey-Kramer multiple comparisons test revealed only the sham group demonstrated a statistically significant improvement in ability to locate the platform goal by the end of the five training days. Nicotine did not provide rats with any benefit in the training phase and rats tended to perform worse than the other groups by the fourth day. The Tukey-Kramer multiple comparisons test showed that on day 5, nicotine treated rats swam a significantly longer distance than sham operated and saline treated rats.

Panel B shows the acquisition data from the 20 trials combined examined post hoc. When collapsing the data to treatment group only, galantamine, nicotine and saline treated rats did not perform as well as sham operated rats, as one-way ANOVA revealed a significant effect of treatment group $F(3, 552) = 14.01, p < 0.0001$. However, galantamine had the lowest path length of the three treatment groups. Furthermore, rats treated with nicotine performed significantly worse than rats treated with galantamine.

The retention phase data for the Morris water maze behavioral task are presented in figure 3.3. Approximately three hours following the completion of the fifth training day, the hidden platform was removed and animals were given 60 seconds to swim. All measurements were taken from the first 15 seconds of the swim. There were no significant differences in path length or total distance traveled, swim speed, number of entries into the target quadrant, time spent in the target quadrant, distance traveled within the target quadrant, or number of crosses over the platform area.

Cortical tissue sparing analysis was performed on nissl stained brain sections. Nissl staining allows for the visualization of neuronal cell bodies protected from injury. Figure 3.4 shows the results of the tissue sparing analysis when comparing galantamine, nicotine, and saline following TBI. The percent cortex spared (mean \pm standard deviation) of the sham operated rats was 104.9 ± 4.3 . A value of approximately 100 percent was expected; however, the current value slightly greater than 100 percent could be explained due to misshaping of the brain upon removal. Percent cortical sparing for the treatment groups undergoing CCI included: saline 80.31 ± 5.40 , galantamine 88.34 ± 8.89 , and nicotine 79.34 ± 10.30 . One-way ANOVA revealed a significant effect of treatment group $F(3, 20) = 14.44, p < 0.0001$. Saline, galantamine, and nicotine treatment following CCI resulted in a significant decrease in percent cortex spared

compared to sham operated rats (Tukey-Kramer multiple comparisons test, $p < 0.05$). Individual rat data are shown in Figure 3.4 to illustrate the variability within each group, which may have accounted for the lack of significant benefit with galantamine treatment. Representative nissl stained sections showing the CCI to the ipsilateral (left) somatosensory cortex are presented for each treatment group (Figure 3.5). Because rats demonstrated high variability within treatment groups, brain sections are representative of rats demonstrating the most cortical sparing for the treatment groups employed.

Quantitative receptor autoradiography was performed in rats administered galantamine, nicotine, or saline for 15 days following TBI. The results for the BTX binding of $\alpha 7$ nAChRs in cortical and hippocampal regions are outlined in Table 3.1. First of all, there was no statistical difference when comparing the ipsilateral side of the brain to the contralateral side of the brain in sham operated rats. Animals subjected to TBI and treated with saline experienced non-significant decreases in BTX binding on the ipsilateral side of the brain compared to the contralateral side of the brain for some regions, and a significant decrease occurred in the hilar layer of the dentate gyrus, $F(1, 24) = 44.40, p < 0.0001$. Following galantamine treatment, no difference was found in ipsilateral BTX binding in any of the hippocampal or cortical regions analyzed compared to the contralateral side of the brain or compared to the ipsilateral brain of sham or saline rats. Moreover, BTX binding in the ipsilateral hilar layer was significantly reduced following TBI with nicotine treatment compared to the contralateral side, $F(1, 24) = 44.40, p < 0.0001$.

The results for the EPI binding of non- $\alpha 7$ nAChRs are presented in Tables 3.2. Comparisons presented are limited to side differences within a treatment group and ipsilateral differences compared to sham or saline treatment groups, unless otherwise noted. There was no difference in EPI binding between the ipsilateral and contralateral sides in sham operated rats for any of the regions tested. TBI demonstrated a significant ipsilateral decrease compared to sham operated rats in the auditory cortex layers 2-3, $F(3, 24) = 6.89, p = 0.0017$, auditory cortex layers 4-6, $F(3, 24) = 16.77, p < 0.0001$, lateral blade of the dentate gyrus, $F(3, 24) = 9.74, p = 0.0002$, medial blade of the dentate gyrus, $F(3, 24) = 6.43, p = 0.0024$, caudate putamen, $F(3, 24) = 7.51, p = 0.001$, thalamus, $F(3, 24) = 5.66, p = 0.0044$, anterodorsal thalamic nucleus/dorsal geniculate nucleus

(ADN/DGN), $F(3, 24) = 4.71, p < 0.0101$, and superior colliculus, $F(3, 24) = 10.65, p = 0.0001$. The auditory cortex layers 2-3, $F(1, 24) = 122.26, p < 0.0001$, lateral blade of the dentate gyrus, $F(1, 24) = 96.60, p < 0.0001$, medial blade of the dentate gyrus, $F(1, 24) = 56.08, p < 0.0001$, thalamus, $F(1, 24) = 64.22, p < 0.0001$, ADN/DGN, $F(1, 24) = 243.25, p < 0.0001$, and superior colliculus, $F(1, 24) = 87.06, p < 0.0001$, also displayed significant reductions compared to the contralateral side of the brain.

TBI caused a significant reduction in non- $\alpha 7$ nAChR binding in galantamine treated rats. Following 15 days of galantamine treatment, EPI binding was significantly reduced in the ipsilateral side compared to the contralateral side of the brain in the auditory cortex layers 2-3, $F(1, 24) = 122.26, p < 0.0001$, lateral blade of the dentate gyrus, $F(1, 24) = 96.60, p < 0.0001$, medial blade of the dentate gyrus, $F(1, 24) = 56.08, p < 0.0001$, thalamus, $F(1, 24) = 64.22, p < 0.0001$, ADN/DGN, $F(1, 24) = 243.25, p < 0.0001$, and superior colliculus, $F(1, 24) = 87.06, p < 0.0001$. In the ipsilateral auditory cortex layers 2-3, $F(3, 24) = 6.89, p = 0.0017$, lateral blade of the dentate gyrus, $F(3, 24) = 9.74, p = 0.0002$, medial blade of the dentate gyrus, $F(3, 24) = 6.43, p = 0.0024$, ADN/DGN, $F(3, 24) = 4.71, p < 0.010$, and the superior colliculus, $F(3, 24) = 10.65, p = 0.0001$, EPI was downregulated compared to the ipsilateral side in sham operated rats. No upregulation in non- $\alpha 7$ nAChRs was apparent, as non- $\alpha 7$ nAChR binding in the contralateral side of the brain was no different than binding in the uninjured side of the brain in saline and sham rats.

A significant decrease in EPI binding was associated with TBI and concomitant nicotine treatment. EPI binding following nicotine treatment was reduced on the ipsilateral side compared to the contralateral side of the brain in auditory cortex layers 2-3, $F(1, 24) = 122.26, p < 0.0001$, auditory cortex layers 4-6, $F(1, 24) = 37.15, p < 0.0001$, lateral blade of the dentate gyrus, $F(1, 24) = 96.60, p < 0.0001$, medial blade of the dentate gyrus, $F(1, 24) = 56.08, p < 0.0001$, caudate putamen, $F(1, 24) = 32.37, p < 0.0001$, thalamus, $F(1, 24) = 64.22, p < 0.0001$, ADN/DGN, $F(1, 24) = 243.25, p < 0.0001$, and superior colliculus, $F(1, 24) = 87.06, p < 0.0001$. In the ipsilateral auditory cortex layers 4-6, $F(3, 24) = 16.77, p < 0.0001$, ADN/DGN, $F(3, 24) = 4.71, p < 0.010$, and superior colliculus, $F(3, 24) = 10.65, p = 0.0001$, EPI was downregulated compared to ipsilateral shams.

Despite the impaired upregulation in multiple brain regions, in the ipsilateral auditory cortex layers 4-6, $F(3, 24) = 16.77, p < 0.0001$, EPI was upregulated compared to shams operated rats. Likewise, EPI binding in the ipsilateral caudate putamen remained significantly higher than in the ipsilateral side of saline treated rats as well as the ipsilateral side of sham operated rats, $F(3, 24) = 7.51, p = 0.001$. Furthermore, EPI demonstrated higher binding in the ipsilateral auditory cortex layers 2-3, $F(3, 24) = 6.89, p = 0.0017$, auditory cortex layers 4-6, $F(3, 24) = 16.77, p < 0.0001$, and lateral blade of the dentate gyrus, $F(3, 24) = 9.74, p = 0.0002$ compared to saline treated rats undergoing TBI, in which EPI had been diminished.

In addition, nicotine treatment significantly enhanced non- $\alpha 7$ nAChR binding in contralateral brain regions tested indicating nicotine's ability to upregulation nAChRs in uninjured brain. EPI binding in the contralateral auditory cortex layers 2-3, $F(3, 24) = 6.89, p = 0.0017$, auditory cortex layers 4-6, $F(3, 24) = 16.77, p < 0.0001$, lateral blade of the dentate gyrus, $F(3, 24) = 9.74, p = 0.0002$, and caudate putamen, $F(3, 24) = 7.51, p = 0.001$, was significantly elevated compared to the contralateral side of sham operated rats.

Study 2. The body mass following TBI in rats treated with galantamine at three different time intervals or saline is shown in figure 3.6. Rats were first weighed immediately prior to receiving a 1.5 mm CCI and again each morning for the next 10 days. Two-way repeated measures ANOVA showed a significant effect of treatment group $F(3, 180) = 4.02, p < 0.0237$, day $F(10, 180) = 72.32, p < 0.0001$, and interaction $F(30, 180) = 4.78, p < 0.0001$. All treatment groups initially lost weight; body mass was significantly reduced on the day following TBI compared to the first study day. This reduction continued through day 6 in the saline treated group and rats treated with galantamine on days 1-2 (GAL1-2). In rats treated with galantamine on days 2-6 (GAL2-6) the weight reduction occurred through study day 7. By contrast, rats treated with galantamine on days 7-11 (GAL7-11) no longer displayed a reduction in body mass on day 4, earlier than the other groups. Of note, baseline weights were elevated in the GAL2-6 and GAL7-11 groups compared to saline treated rats. On study day 7 in which Morris water maze testing began, the GAL7-11 group had an increased body mass

compared to all other groups. In addition, on the last study day, the GAL2-6 and GAL7-11 groups weighed more than saline treated rats.

The results of the acquisition training in the Morris water maze behavioral task for rats treated with galantamine at different time intervals following CCI is presented in figure 3.7. Panel A shows the training phase of the Morris water maze task. Each rat performed four swim trials on each of the five test days. The path length traveled to find the platform was used for analysis instead of latency to platform goal to avoid differences in swim speed. For comparative purposes, data from a sham operated group of rats (from the previous study) was included in the analysis. Two-way repeated measures, ANOVA revealed a significant effect of treatment group $F(4, 444) = 4.61, p = 0.0018$. The Tukey-Kramer multiple comparisons test demonstrated on days 1, 4 and 5, there were no differences between groups. On day 2, the saline and GAL7-11 groups swam farther than the sham operated rats. By day 3, the saline and GAL1-2 groups swam farther than sham operated rats. There was also a significant effect of day $F(4, 444) = 28.62, p < 0.0001$. The GAL2-6, saline, and sham operated groups all performed better by day five, but the GAL1-2 and GAL7-11 groups did not improve by the end of the training phase compared to the first day of the trial.

Panel B shows the acquisition data from the 20 trials combined. When collapsing the data to compare treatment group only, one-way ANOVA revealed a significant effect of treatment group $F(4, 569) = 7.65, p < 0.0001$. The GAL1-2 and saline groups demonstrated a significantly longer path length compared to sham operated rats.

A further post hoc analysis of Morris water maze performance examined the fourth daily trial of training, as each day of learning contained four separate trials in which variability may be high (figure 3.8). Data shown are the path lengths traveled to find the hidden platform from the fourth trial of days 1-5. Data from a sham operated group of rats (from the previous study) was included in the analysis. The GAL2-6 group displayed the lowest mean path length with the exception of the sham group.

The retention phase data for the Morris water maze behavioral task are presented in figure 3.9. Approximately three hours following the completion of the fifth training day, the hidden platform was removed and animals were given 60 seconds to swim. All measurements were taken from the first 15 seconds of the swim. One-way ANOVA and

the Tukey-Kramer procedure revealed a significant effect of treatment group for total distance traveled $F(4, 24) = 12.88, p < 0.0001$ and speed $F(4, 24) = 12.95, p < 0.0001$. All treatment groups traveled a shorter distance and swam slower than the sham group (Figure 3.9A, B). A significant treatment effect was demonstrated for the distance traveled within the target quadrant $F(4, 24) = 5.71, p = 0.0022$. The GAL7-11 and saline groups swam a significantly shorter distance within the target quadrant compared to sham operated rats. The GAL2-6 group displayed the highest group mean only second to sham rats (Figure 3.9E). There were no significant differences in number of entries into the target quadrant, time spent in the target quadrant, or number of crosses over the platform area.

The results of the cortical tissue sparing analysis are reported in Figure 3.10. When comparing galantamine at various dosing intervals following TBI, The GAL1-2, GAL2-6, and GAL7-11 groups all showed increased tissue sparing compared to saline treated rats, but none of these protective effects was statistically significant. The percent cortex spared values (mean \pm standard deviation) were $89.89 \pm 8.58, 92.73 \pm 10.05, 91.02 \pm 11.06, 89.35 \pm 11.15$ for the saline, GAL1-2, GAL2-6, and GAL7-11 groups, respectively. The remaining variability and low sample size were limitations of this analysis. Representative nissl stained sections showing the CCI to the ipsilateral (left) somatosensory cortex are presented for each treatment group (Figure 3.11). Brain sections are representative of rats demonstrating the most cortical sparing for the treatment groups employed.

Discussion

Ongoing research has suggested galantamine's facilitative actions as an APL at nAChRs may lead to its potential neuroprotective effects. Due to findings from our laboratory in experimental TBI and the abundance of literature supporting the association between cognitive impairment and deficits in nAChR expression, namely in AD, we hypothesized that nAChRs are important mediators of cell death and survival pathways in the hippocampus and cortex. An evaluation of the effects of galantamine on nAChR

expression and cognition in rats following experimental brain injury was conducted herein.

Quantitative receptor autoradiography was performed to determine the effects of galantamine treatment on $\alpha 7$ and non- $\alpha 7$ nAChR densities following TBI. The results of the binding analyses show that pharmacological modulation of nAChRs with either galantamine or nicotine was unable to reduce the TBI induced deficit in non- $\alpha 7$ nAChR density in the current paradigm. A summary of the binding results shows the number of ipsilateral brain regions significantly altered following experimental TBI (Table 3.3).

In the saline treated group, a significant decrease was seen in the hilar layer of the dentate gyrus as previous studies have shown (Verbois et al., 2000; Verbois et al., 2002). BTX binding in the ipsilateral hilar layer was significantly reduced following nicotine treatment compared to the contralateral side as well. This result is also consistent with previous research in that nicotine does not upregulate $\alpha 7$ nAChRs at the current dose (Verbois et al., 2003b).

Results from this receptor binding analysis suggest that nicotine and galantamine affect non- $\alpha 7$ receptors to a greater degree than $\alpha 7$ receptors at least when used in the current treatment paradigm. The results of the EPI binding analysis demonstrate a mild to moderate form of experimental TBI impairs the ability of agonist induced receptor upregulation. Following TBI and saline treatment, decreases were found in all brain regions. Galantamine treatment resulted in significant reductions and did not result in increases in EPI binding in the contralateral brain as expected. Nicotine treatment was associated with a downregulation in ipsilateral EPI binding. In addition, nicotine treatment significantly enhanced non- $\alpha 7$ nAChR binding in some contralateral brain regions tested indicating nicotine's ability to upregulation nAChRs in uninjured brain. These finding suggests that upregulation of non- $\alpha 7$ nAChR by galantamine and nicotine is altered by TBI.

Upregulation of nAChRs following agonist stimulation has been well documented. Chronic nicotine exposure in rodents (Marks et al., 1983; Schwartz and Kellar, 1983) and in the postmortem brains of smokers (Benwell et al., 1988; Nyback et al., 1989; Wonnacott, 1990; Breese et al., 1997a) results in the upregulation of nAChRs, and agonist induced nAChR upregulation is thought to be due to post-translational

mechanisms (Marks et al., 1992; Pauly et al., 1996; Gentry and Lukas, 2002). Additionally, the pattern of upregulation by nicotine has been shown to depend on receptor subtype. For example, $\alpha 6\beta 4$ nAChRs upregulate at different doses and exposure times compared to $\alpha 4\beta 2$ nAChRs (Walsh et al., 2008). Recently, rats receiving a choline supplemented diet for two weeks demonstrated a selective upregulation in $\alpha 7$ nAChRs in cortical and hippocampal brain regions (Guseva et al., 2006). Physiologic changes occurring in TBI such as altered cerebral blood flow could affect the concentration, distribution, and pharmacodynamic properties of nAChR agonists within various brain regions. TBI may interfere with any one of the processes involved in nAChR upregulation including transcription, receptor subunit assembly, post-translational modifications or desensitization. Evaluation of mRNA expression would help to characterize TBIs effect on upregulation in this model.

For the current study, the dose of 3.3 mg/kg twice daily was chosen based on the pharmacokinetic and pharmacodynamic literature, behavioral observations following dosing in preliminary studies, and the previous use of twice daily dosing regimens by our laboratory. Within minutes of dosing, rats displayed several physical indicators that the drug reached the systemic circulation and distributed into the brain including salivation, lip smacking, and yawning. Yawning has been used as an indicator of AChEI activity within the CNS (Ogura et al., 2001). A subcutaneous dose of 3 mg/kg in rats results in peak brain concentrations of greater than 1 μ M within one hour in which galantamine acts as an open channel blocker (Sharp et al., 2004). A narrow therapeutic window occurs following dosing when concentrations fall into a range that is optimal for APL activity (Geerts et al., 2005).

Galantamine is metabolized by a number of mechanisms including hepatic metabolism through cytochrome P450, glucuronidation, and renal excretion of the unchanged parent compound (Mannens et al., 2002). Cytochrome P450 isoforms CYP2D6 and CYP3A4 are involved in galantamine's metabolism in humans (Ortho-McNeil Neurologics, 2008) and animals undergo similar metabolic pathways (Mannens et al., 2002). In humans (Shedlofsky et al., 1994) and in rats (Roe et al., 1998), exogenous administration of lipopolysaccharide induces an acute phase response characterized by inflammation producing fever and elevated cytokines that results in

depressed cytochrome P450 activity. Studies have shown that cytochrome P450 expression is altered following experimental TBI in the rat. In one study, a cerebral percussive injury resulted in a downregulation of CYP3A mRNA at 24 hours, without changes in protein levels or enzyme activity at 24 or 48 hours post injury (Toler et al., 1993). In another study (Kalsotra et al., 2003), rats subjected to a controlled cortical impact (at a higher severity level than in the study herein) demonstrated a reduction in total hepatic cytochrome P450 content at 24 hours followed by an induction two weeks later. In this study, TBI did not alter CYP3A protein levels but rather enhanced activity 24 hours after injury and increased both protein expression and activity two weeks following injury; no change was found in the CYP2D isoform. Any alterations in drug metabolism may affect galantamine's brain concentrations, but since galantamine undergoes multiple routes of elimination, the full impact of this is unknown. Furthermore, the elimination half-life of galantamine is only 40-50 minutes in rats (Mihailova and Yamboliev, 1986). Therefore, twice daily dosing should not result in steady-state concentrations; thus, small increases or decreases in the concentration would not prevent achievement of the target concentration after each dose. It is presumed that brain concentrations reached the target levels to facilitate APL activity in this study; however, dosing more often than twice daily may improve the study design. Hence, the dosing regimen ideal for studies assessing galantamine's modulatory affect on nAChRs following TBI may require further consideration and validation through measurement of brain concentrations.

In other models, galantamine induced upregulation of nAChRs has been described. A mouse model that overexpresses acetylcholinesterase (Svedberg et al., 2004) is used to study the effects of compensatory mechanisms resulting from reduced acetylcholine in the synapse which causes upregulation of acetylcholine receptors and deficits in spatial learning and memory. This model can be used to study situations in which cholinergic transmission is deficient. Galantamine 2 mg/kg given subcutaneously twice daily for 10 days caused an upregulation in [³H]-cytisine binding in hippocampus and BTX binding in the cortex of control mice but not AChE overexpressing mice (in which receptor expression was already upregulated) (Svedberg et al., 2004). Rabbits treated with galantamine also showed enhanced nAChR expression and improved

learning and memory in young (Woodruff-Pak et al., 2001) and old (Woodruff-Pak and Santos, 2000; Woodruff-Pak et al., 2001) individuals. Donepezil also demonstrated this benefit but only in old rabbits. When tested in the young, no change in EPI binding was determined; however, galantamine and donepezil could reverse the cognitive impairment caused by mecamylamine, a non-competitive nAChR antagonist (Woodruff-Pak et al., 2003). A positron emission tomography study of galantamine treatment, 16-24 mg/day, in AD patients displayed a 30-40 percent inhibition of AChE and no changes in [¹¹C]-nicotine binding in the cortex from three weeks to 12 months of treatment (Kadir et al., 2008). However, the authors found a correlation of increasing galantamine concentrations in the plasma and cortical [¹¹C]-nicotine binding.

Galantamine's cognitive enhancing effects have been evaluated in both *in vitro* and *in vivo* models. In mice receiving a lesion to the nucleus basalis magnocellularis which results in cholinergic dysfunction, galantamine i.p. four hours before testing attenuated deficits in a passive avoidance and modified Morris water maze tests (3.0 mg/kg and 2.0 mg/kg were optimal doses, respectively). But galantamine impaired performance in sham mice (Sweeney et al., 1990). In C57B1/10 mice galantamine enhanced Morris water maze performance when given at low doses (0.1-1.0 mg/kg), 30 minutes prior to testing (Vincent et al., 1988). Galantamine when given in a single i.p. dose following reperfusion after transient forebrain ischemia (carotid artery occlusion) to rats resulted in improved learning ability using an active avoidance task called the shuttle-box test (Iliev et al., 2000).

Older F344 rats 22 months of age receiving continuous infusions of galantamine (0.277 mg/day resulting in 60 percent AChE inhibition) demonstrated enhanced nicotine nAChR upregulation in cortex and hippocampus but no change in the spatial working memory task in the radial arm maze; donepezil, an AChEI without APL activity, showed the same results (Barnes et al., 2000). In aged Fisher 344 rats 15 days of subcutaneous galantamine upregulated cortical nAChRs demonstrated by EPI binding and improved spatial learning in the Morris water maze and light/dark box tasks. However, donepezil caused the same result (Hernandez et al., 2006).

In the current study, galantamine's potential cognitive enhancing properties were evaluated in experimental brain injury. Unfortunately, galantamine did not enhance

cognition as we expected. The first time galantamine treatment following TBI was tested in the Morris water maze, rats appeared to perform better than the saline and nicotine treated animals on the first two days of the acquisition phase. However, this effect did not continue throughout the learning trials in which galantamine treated rats learned similar to saline treated rats. When combining all trials, galantamine demonstrated the lowest path length, but this was not statistically significant.

Because of the lack of statistically significant improvements in cognitive function, galantamine was tested at different time intervals following TBI in order to attempt to optimize outcomes, as one *in vitro* study concluded that a longer exposure to galantamine resulted in diminished function in the nAChR (Barik et al., 2005). In the examination of galantamine treatment administered at different time periods following TBI, those rats in the GAL2-6 group performed better than any other group although this was not statistically significant. Furthermore, this group did not receive galantamine on the Morris water maze testing days. In the first galantamine study, dosing was continued throughout the behavioral testing days. The test should be repeated so that dosing is ended before the initiation of behavioral testing as this may improve outcomes. Galantamine did not show any benefit in the probe trial. This may be due to the short duration between completing the learning phase and initiating the retention aspect of the task. In addition, only the first 15 seconds of a total of 60 seconds available for the rat to search for the platform in the probe trial was analyzed. This was due to the belief that learned rats will not continue to search for the platform in its presumed location if it is not there; however, this strategy did not show significant results. The lack of statistical significance in the behavioral testing may be remedied by increasing the sample size as there was considerable variability in the data for this task. In addition, saline treated rats and sham operated rats demonstrated increases in body weight compared to baseline during the behavioral trial of the first galantamine treatment study and this may have affected the results. In the second galantamine analysis, the GAL7-11 group displayed an increase in body mass. This factor probably did not influence the acquisition portion of the Morris water maze behavioral task which used the distance traveled as the outcome measure which does not depend on swim speed. However, this may have impacted the probe analyses.

No significant increases in cortical tissue sparing were detected. Due to a high variability in sham subjects as well as the large treatment group variability, a larger sample size may have been warranted to produce statistically significant results. Further studies with galantamine including pretreatment and dose adjustments may help to elucidate galantamine's full potential benefit.

In a previous study, nicotine administered i.p. twice daily for 11 days following TBI partially attenuated deficits in the training phase (distance traveled to the platform) of the Morris water maze behavioral task. Nicotine treated rats performed as well as sham operated rats on days 1, 2, and 3 of 5 days of testing as well as in a retention probe test in the Morris water maze behavioral task (Verbois et al., 2003b). In the current study, nicotine did not enhance cognition as our laboratory has previously shown. The lack of effect is likely due to the known psychostimulant properties of nicotine administration which was continued on the days of behavioral testing in this study unlike the previous study. Such behavioral effects may impede the animals' ability to perform optimally.

In summary, the analysis of galantamine following mild to moderate TBI suggests brain injury interferes with the pharmacologically mediated upregulation of nAChRs. Galantamine and nicotine administration did not result in significant and widespread elevations in non- $\alpha 7$ nAChRs that would be expected following agonist stimulation. The mechanism by which TBI is involved is unknown but may involve the regulation of mRNA expression, and thus, investigations into the transcriptional regulation of nAChRs in TBI are needed. Since galantamine partially remediated TBI induced cognitive declines at early, but not later stages of acquisition testing, the kinetics and timing of drug delivery may require additional considerations including the testing of galantamine in a pretreatment paradigm. Further studies are needed to fully elucidate galantamine's potential benefit in traumatic brain injury and other models of cholinergic dysfunction.

Table 3.1. $\alpha 7$ nAChR density, as measured by α -[¹²⁵I]-bungarotoxin (BTX) binding, in hippocampal and cortical brain regions following TBI and 15 days of drug treatment. Data shown are the amount of binding (nCi/mg wet tissue) group means \pm standard deviation.

Brain Region	Sham, Saline		CCI, Saline		CCI, Galantamine		CCI, Nicotine	
	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral
Neocortex								
CTX (1-4)	0.38 \pm 0.05	0.38 \pm 0.05	0.36 \pm 0.06	0.34 \pm 0.08	0.35 \pm 0.06	0.38 \pm 0.07	0.39 \pm 0.09	0.39 \pm 0.10
CTX (5-6)	0.71 \pm 0.09	0.70 \pm 0.08	0.67 \pm 0.07	0.67 \pm 0.15	0.63 \pm 0.09	0.67 \pm 0.12	0.73 \pm 0.11	0.74 \pm 0.13
Hippocampus								
Stratum Oriens	0.73 \pm 0.12	0.75 \pm 0.10	0.77 \pm 0.16	0.75 \pm 0.13	0.70 \pm 0.14	0.65 \pm 0.09	0.78 \pm 0.17	0.71 \pm 0.18
CA1 layer	0.25 \pm 0.06	0.23 \pm 0.04	0.26 \pm 0.05	0.26 \pm 0.05	0.26 \pm 0.05	0.25 \pm 0.06	0.29 \pm 0.08	0.27 \pm 0.06
CA3 layer	0.54 \pm 0.10	0.54 \pm 0.11	0.62 \pm 0.12	0.50 \pm 0.07	0.58 \pm 0.06	0.53 \pm 0.10	0.66 \pm 0.14	0.58 \pm 0.15
DG (lateral)	0.50 \pm 0.05	0.48 \pm 0.07	0.50 \pm 0.06	0.43 \pm 0.04	0.51 \pm 0.05	0.45 \pm 0.04	0.49 \pm 0.11	0.44 \pm 0.07
DG (hilar)	1.39 \pm 0.10	1.36 \pm 0.12	1.43 \pm 0.11	1.30 \pm 0.06*	1.48 \pm 0.08	1.40 \pm 0.06	1.50 \pm 0.08	1.37 \pm 0.12*

* denotes significantly different from the contralateral side

Statistical significance determined at $\alpha = 0.05$

Abbreviations: CTX, auditory cortex layers 1-4 or 5-6; DG, lateral or hilar blade of the dentate gyrus

Table 3.2. The effects of traumatic brain injury and nicotinic receptor drug treatment on non- $\alpha 7$ nAChRs throughout the brain following TBI. Non- $\alpha 7$ nAChR expression was measured by [125 I]-epibatidine (EPI) binding. Data shown are the amount of binding (nCi/mg wet tissue) group means \pm standard deviation.

Brain Region	Sham, Saline		CCI, Saline		CCI, Galantamine		CCI, Nicotine	
	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral
Neocortex								
CTX (2-3)	0.19 \pm 0.03	0.18 \pm 0.02	0.18 \pm 0.02	0.13 \pm 0.03* Ψ	0.19 \pm 0.03	0.15 \pm 0.02* Ψ	0.25 \pm 0.02#	0.18 \pm 0.05* \dagger
CTX (4-6)	0.14 \pm 0.02	0.13 \pm 0.01	0.13 \pm 0.02	0.11 \pm 0.02 Ψ	0.13 \pm 0.01	0.12 \pm 0.01	0.20 \pm 0.02#	0.17 \pm 0.04* \dagger Ψ
Basal ganglia								
Caudate putamen	0.20 \pm 0.02	0.19 \pm 0.02	0.19 \pm 0.02	0.17 \pm 0.02 Ψ	0.20 \pm 0.02	0.19 \pm 0.02	0.24 \pm 0.02#	0.22 \pm 0.03* \dagger Ψ
Diencephalon								
Thalamus (AV/VL)	0.63 \pm 0.02	0.60 \pm 0.07	0.62 \pm 0.04	0.45 \pm 0.08* Ψ	0.61 \pm 0.05	0.47 \pm 0.07*	0.72 \pm 0.07	0.53 \pm 0.10*
ADN/DGN	1.07 \pm 0.08	1.05 \pm 0.05	1.09 \pm 0.03	0.88 \pm 0.04* Ψ	1.06 \pm 0.07	0.82 \pm 0.12* Ψ	1.09 \pm 0.05	0.83 \pm 0.07* Ψ
Hippocampus								
DG (lateral)	0.06 \pm 0.01	0.06 \pm 0.01	0.06 \pm 0.00	0.05 \pm 0.01* Ψ	0.06 \pm 0.01	0.05 \pm 0.01* Ψ	0.08 \pm 0.01#	0.06 \pm 0.01* \dagger
DG (medial)	0.13 \pm 0.01	0.13 \pm 0.02	0.11 \pm 0.01	0.08 \pm 0.01* Ψ	0.12 \pm 0.03	0.09 \pm 0.03* Ψ	0.15 \pm 0.02	0.11 \pm 0.02*
Mesencephalon								
Superior colliculus	0.69 \pm 0.07	0.70 \pm 0.05	0.68 \pm 0.04	0.52 \pm 0.07* Ψ	0.61 \pm 0.05	0.49 \pm 0.05* Ψ	0.69 \pm 0.05	0.54 \pm 0.08* Ψ

* denotes significantly different from the contralateral side

\dagger denotes significantly different from the ipsilateral side of CCI, saline rats

Ψ denotes significantly different from the ipsilateral side of sham operated rats

denotes significantly different from the contralateral side of sham operated rats

Statistical significance determined at $\alpha = 0.05$

Abbreviations: CTX, auditory cortex layers 1-4 or 5-6; AV, anteroventral thalamic nucleus; VL, ventrolateral thalamic nucleus; ADN, Anterodorsal thalamic nucleus; DGN, dorsal geniculate nucleus; DG, lateral or hilar blade of the dentate gyrus

Table 3.3. A summary of the binding results shows the number of ipsilateral brain regions significantly altered following experimental TBI. Decrease columns represent the number of regions displaying significant reductions when comparing the ipsilateral side to the contralateral side of the brain and/or the ipsilateral brain of sham rats. Increase columns represent the number of regions displaying significant elevations when comparing the ipsilateral side to the saline and/or sham group. A total of seven and eight brain regions were analyzed in the BTX and EPI binding studies, respectively.

Binding Study	Sham, Saline		CCI, Saline		CCI, Galantamine		CCI, Nicotine	
	Decrease	Increase	Decrease	Increase	Decrease	Increase	Decrease*	Increase
BTX	0/7	0/7	1/7	0/7	0/7	0/7	1/7	0/7
EPI	0/8	0/8	8/8	0/8	6/8	0/8	8/8	4/8

* EPI binding in the nicotine treatment group resulted in upregulation on the contralateral side in 4 of the 8 brain regions showing an ipsilateral decrease above
 Abbreviations: BTX, α -[¹²⁵I]-bungarotoxin; EPI, [¹²⁵I]-epibatidine

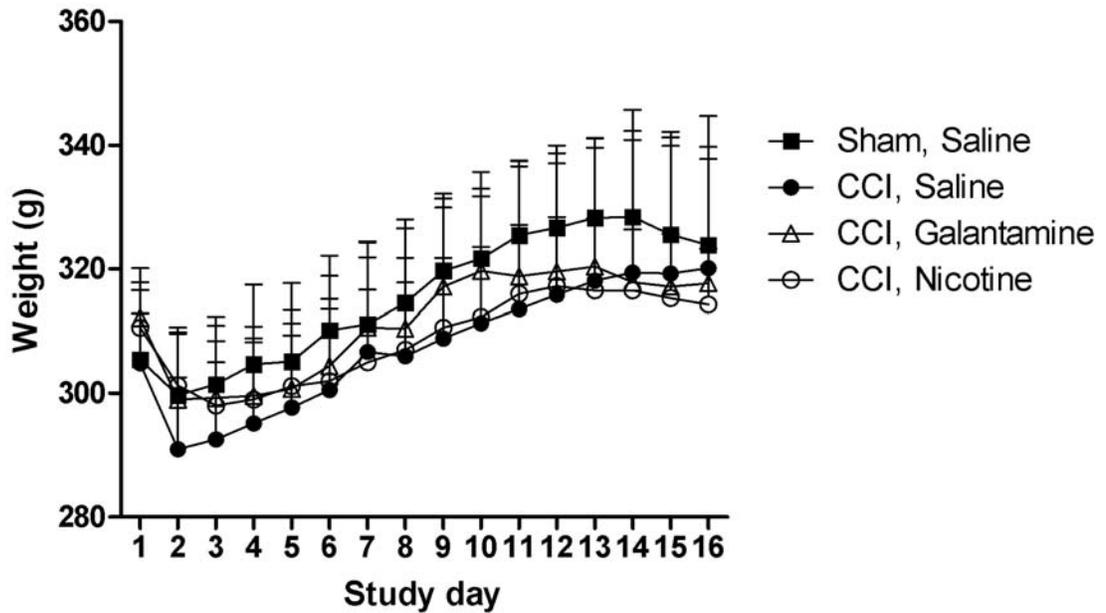


Figure 3.1. Body mass following TBI in rats treated with galantamine, nicotine or saline. Rats were first weighed immediately before receiving a 1.5 mm controlled cortical impact (CCI), the morning of study day 1. Rats were weighed again each morning before receiving the study drugs (beginning on day 2) for the next 15 days. Drug treatments included twice daily intraperitoneal injections of 3.3 mg/kg galantamine (n=7), 0.3 mg/kg nicotine (n=7), or normal saline vehicle (n=7). The sham surgery group (n=7) was administered saline. Symbols and bars represent group means, weight in grams (g), and standard deviation for each day. Data were analyzed with a two-way (treatment group x day) repeated measures ANOVA, followed by a Tukey-Kramer multiple comparison test. A significant effect of day $F(15, 360) = 54.35, p < 0.0001$ was detected.

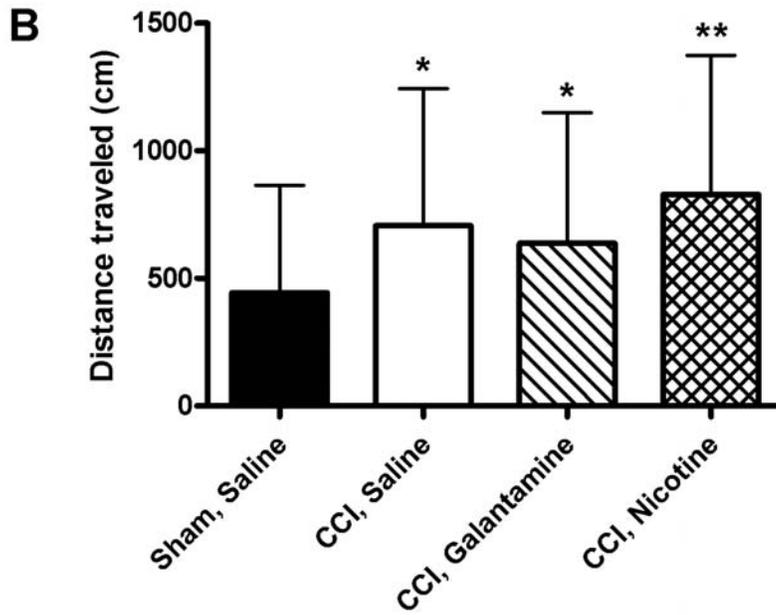
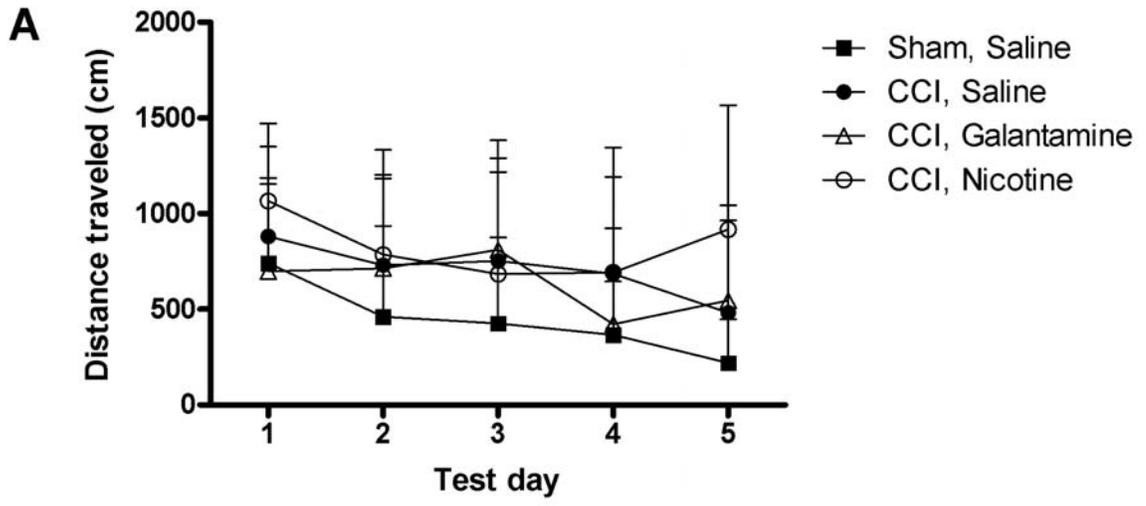


Figure 3.2. Acquisition training in the Morris water maze behavioral task in rats treated with galantamine, nicotine or saline. Panel A shows the training phase of the Morris water maze, performed on five consecutive days, beginning eleven days following injury or sham operation (study day 12). Animals were given 60 seconds to find the hidden platform. The path length, in centimeters, traveled to find the platform was recorded. If the rat did not find the platform, the path length for the 60 second swim was obtained. Each rat performed four swim trials on each of the five test days. Drug treatments were administered 24 hours following injury and continued through the behavioral task for a total of 15 days. Two-way repeated measures ANOVA revealed a significant effect of treatment group $F(3, 432) = 8.54, p < 0.0001$, day $F(4, 432) = 9.12, p < 0.0001$, and interaction $F(12, 432) = 2.15, p = 0.0135$. Panel B shows the acquisition data from the 20 trials combined. One-way ANOVA revealed a significant effect of treatment group $F(3, 552) = 14.01, p < 0.0001$. Data are expressed as mean and standard deviation. * denotes a significant difference compared to sham operated rats; ** denote a significant difference compared to the CCI, galantamine group and sham operated rats (Tukey-Kramer multiple comparisons test, $p < 0.05$).

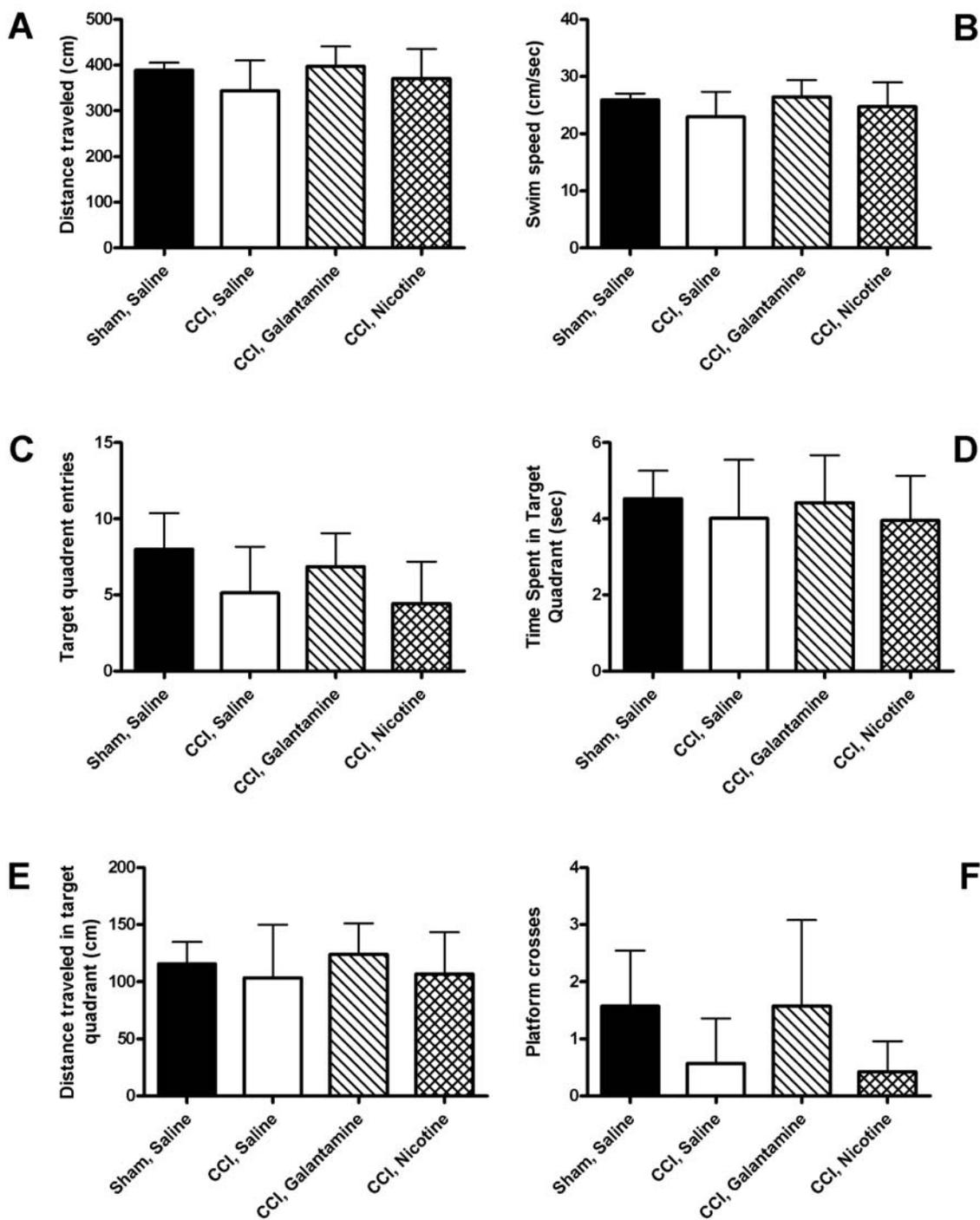


Figure 3.3. Retention in the Morris water maze behavioral task in rats treated with galantamine, nicotine or saline. Approximately three hours following the completion of the fifth training day, the hidden platform was removed and animals were given 60 seconds to swim. All measurements were taken from the first 15 seconds of the swim. Data are as follows: A, path length or total distance traveled (cm); B, swim speed (cm/sec); C, number of entries into the target quadrant; D, distance traveled within the target quadrant (cm); E, number of crosses over the platform area; F, time spent over the platform area (sec). Data are expressed as mean and standard deviation. Abbreviations: cm, centimeters; sec, seconds

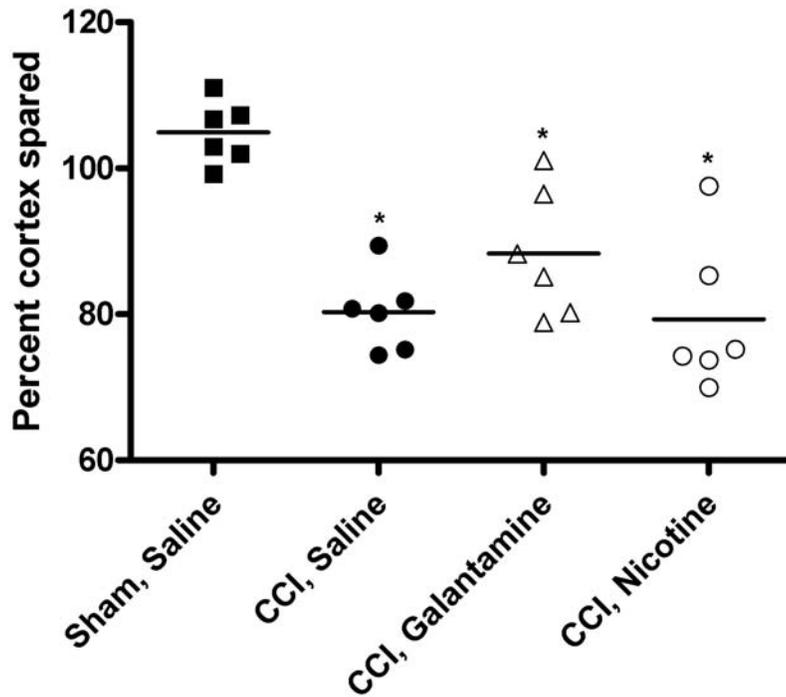


Figure 3.4. Cortical tissue sparing analysis was performed on eleven equally spaced brain sections following nissl staining. The cortical area was measured on each side of the brain separately. The percentage of cortical tissue spared was calculated by dividing the mean cortical area for the ipsilateral hemisphere by the mean cortical area for the contralateral side of the brain, multiplied by 100. One-way ANOVA revealed a significant effect of treatment group $F(3, 20) = 14.44, p < 0.0001$. Individual data are shown for each group; bars represent the group mean. * denotes a significant difference compared to sham operated rats (Tukey-Kramer multiple comparisons test, $p < 0.05$).



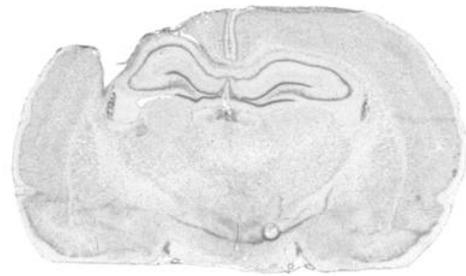
Sham, Saline



CCI, Saline



CCI, Galantamine



CCI, Nicotine

Figure 3.5. Representative nissl stained sections are shown from the cortical sparing analysis (Figure 3.4) in rats following CCI. Note the cortical cavitation present on the ipsilateral (left) somatosensory cortex of the CCI, saline rat.

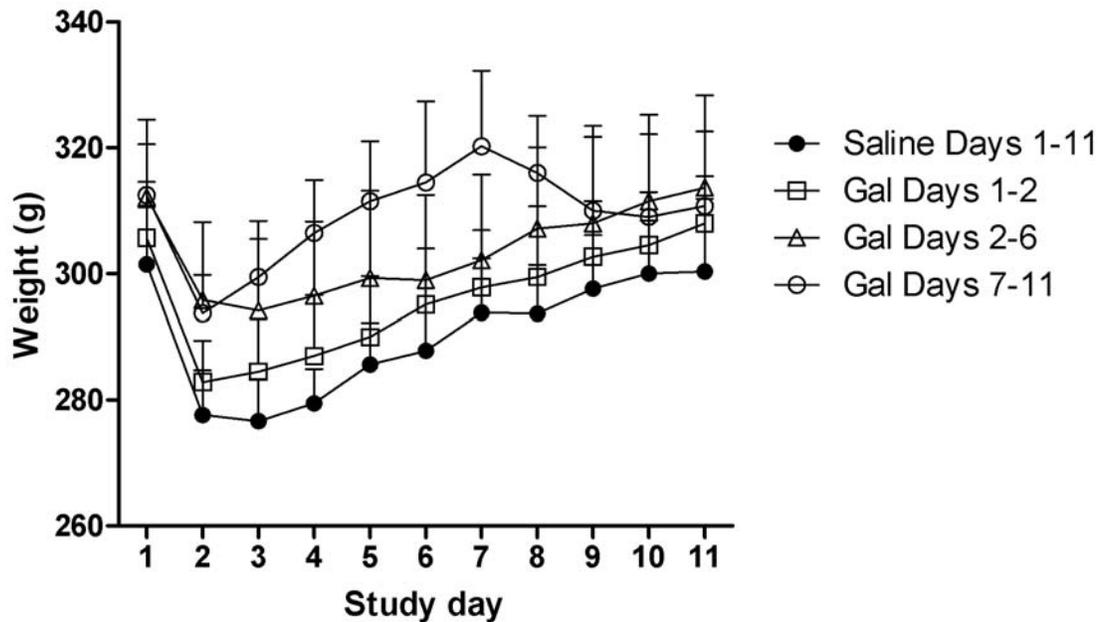


Figure 3.6. Body mass following TBI in rats treated with galantamine at three different time intervals or saline. Rats were first weighed immediately prior to receiving a 1.5 mm controlled cortical impact (CCI) on the morning of study day 1. Rats were weighed again each morning for the next 10 days. Drug treatment groups included twice daily intraperitoneal injections of galantamine 3.3 mg/kg on days 1-2 (n=6), days 2-6 (n=6), days 7-11 (n=4), or saline days 1-11 (n=6). Rats randomized to the galantamine days 1-2 group received their first injection 30 minutes following CCI. Symbols and bars represent group means, weight in grams (g), and standard deviation for each day. Data were analyzed with a two-way (treatment group x day) repeated measures ANOVA, followed by a Tukey-Kramer multiple comparison test. A significant effect of treatment group $F(3, 180) = 4.02, p=0.0237$, day $F(10, 180) = 72.32, p<0.0001$, and interaction $F(30, 180) = 4.78, p<0.0001$ was detected. Abbreviation: GAL, galantamine

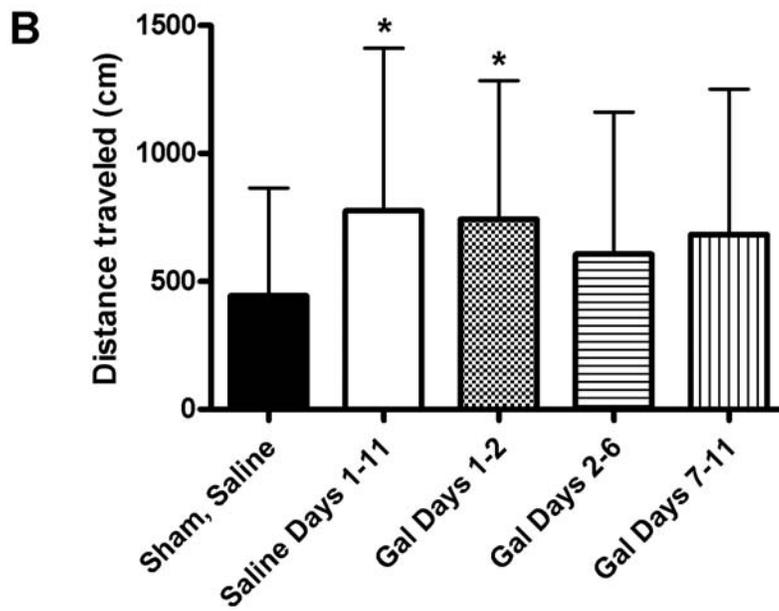
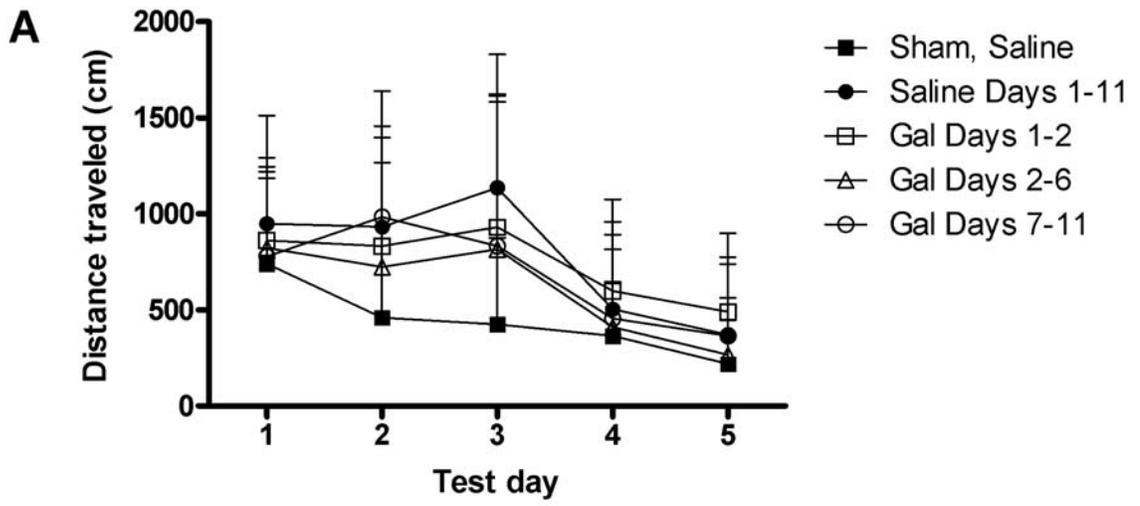


Figure 3.7. Acquisition training in the Morris water maze behavioral task in rats treated with galantamine at three different time intervals or saline. Panel A shows the training phase of the Morris water maze task which was performed on five consecutive days, beginning six days following injury (study day 7). Animals were given 60 seconds to find the hidden platform. The path length, in centimeters, traveled to find the platform was recorded. If the rat did not find the platform, the path length for the 60 second swim was obtained. Each rat performed four swim trials on each of the five test days. Two groups of rats received drug treatments during the five days of behavior testing, rats receiving galantamine on days 7-11 and saline treated rats. Data from a sham operated group of rats (from a previous study) were included in the analysis. Two-way repeated measures ANOVA revealed a significant effect of treatment group $F(4, 444) = 4.61$, $p=0.0018$ and day $F(4, 444) = 28.62$, $p<0.0001$. Panel B shows the acquisition data from the 20 trials combined. One-way ANOVA revealed a significant effect of treatment group $F(4, 569) = 7.65$, $p<0.0001$. Data are expressed as mean and standard deviation. * denotes a significant difference compared to sham operated rats (Tukey-Kramer multiple comparisons test, $p<0.05$). Abbreviation: GAL, galantamine

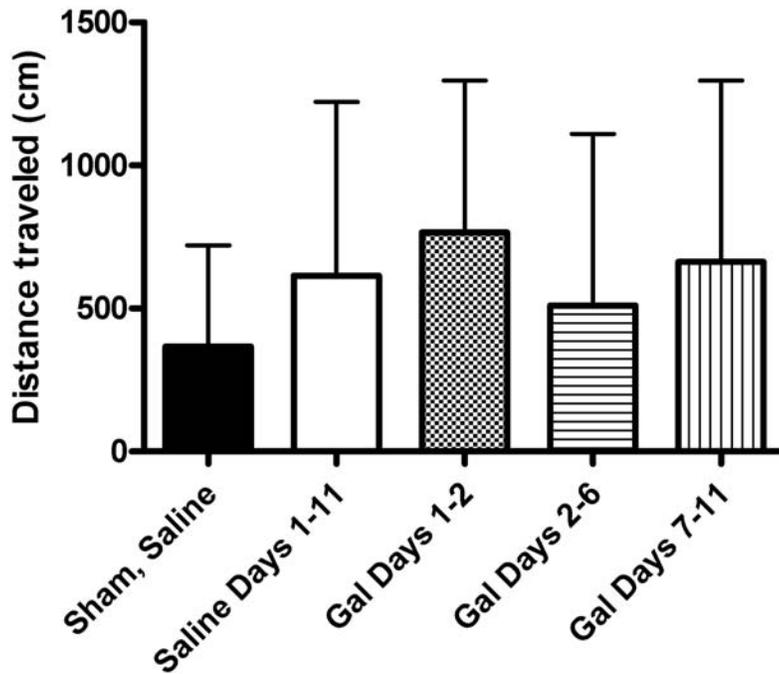


Figure 3.8. Fourth trial of acquisition training in the Morris water maze in rats treated with galantamine at three different time intervals or saline. The training phase of the Morris water maze task was performed on five consecutive days, beginning six days following injury (study day 7). Each rat performed four swim trials on each of the five test days. Data presented are path length (mean and standard deviation in centimeters) traveled to find the hidden platform from the fourth trial on days 1-5. Two groups of rats received drug treatments during the five days of behavior testing, rats receiving galantamine on days 7-11 and saline treated rats. Data from a sham operated group of rats (from a previous study) were included in the analysis. Abbreviation: GAL, galantamine

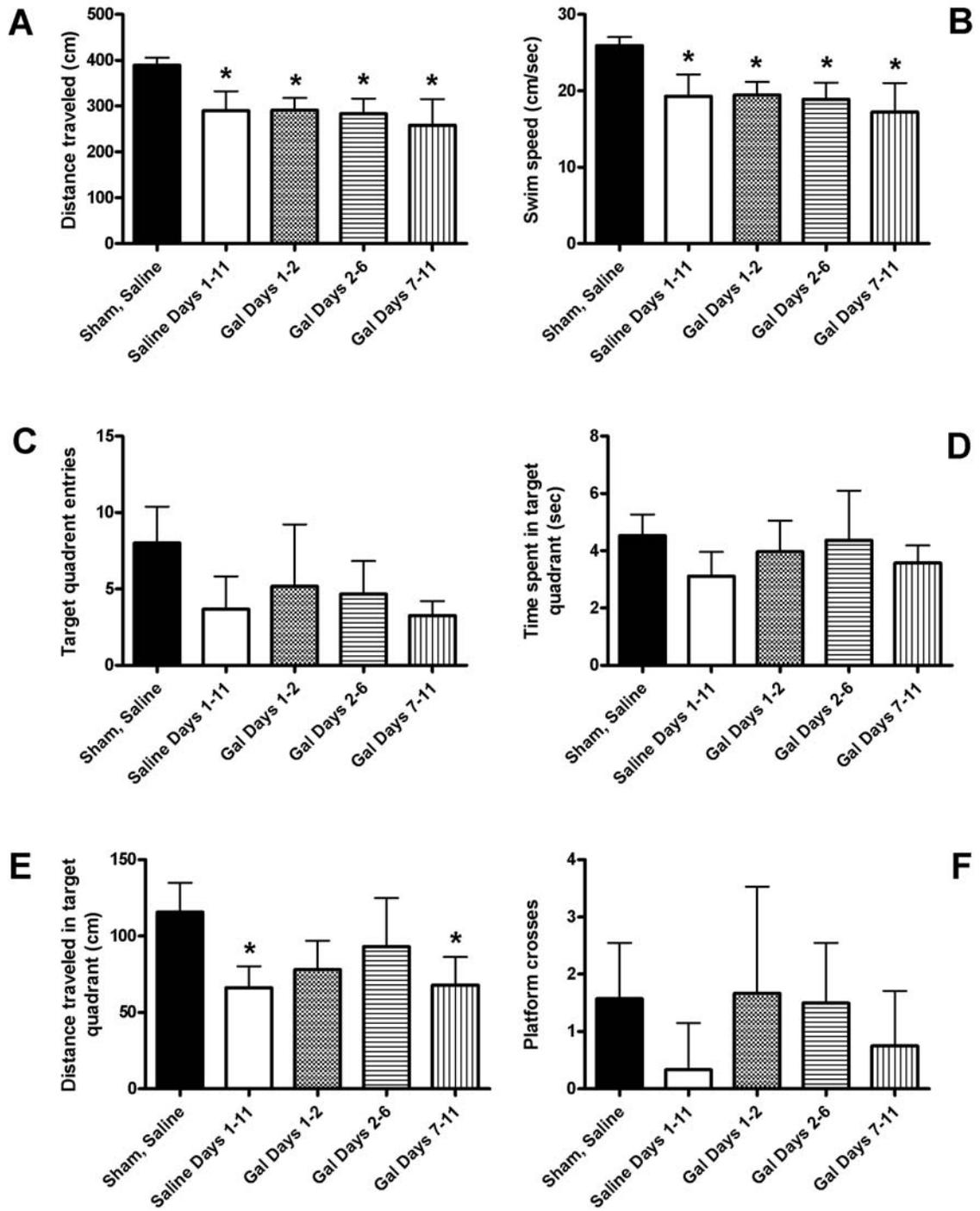


Figure 3.9. Retention in the Morris water maze behavioral task in rats treated with galantamine at three different time intervals or saline. Approximately three hours following the completion of the fifth training day, the hidden platform was removed and animals were given 60 seconds to swim. All measurements were taken from the first 15 seconds of the swim. Data are as follows: A, path length or total distance traveled (cm); B, swim speed (cm/sec); C, number of entries into the target quadrant; D, distance traveled within the target quadrant (cm); E, number of crosses over the platform area; F, time spent over the platform area (sec). Data from a sham operated group of rats (from a previous study) were included in the analysis. Data are expressed as mean and standard deviation. One-way ANOVA revealed a significant effect of treatment group for total distance traveled $F(4, 24) = 12.88, p < 0.0001$, speed $F(4, 24) = 12.95, p < 0.0001$, and distance traveled within the target quadrant $F(4, 24) = 5.71, p = 0.0022$. Data are expressed as mean and standard deviation. * denotes a significant difference compared to sham operated rats (Tukey-Kramer, multiple comparisons test, $p < 0.05$). Abbreviations: GAL, galantamine; cm, centimeters; sec, seconds

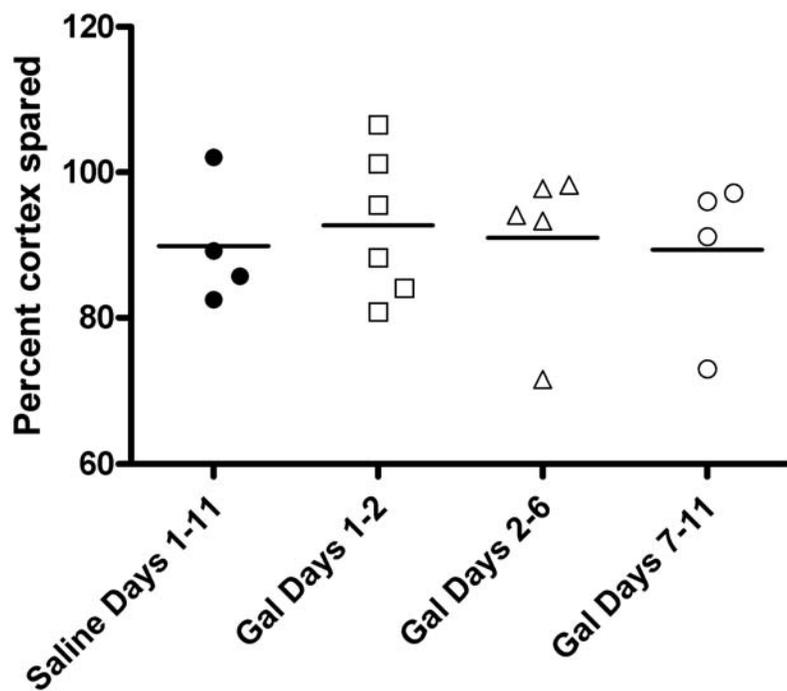
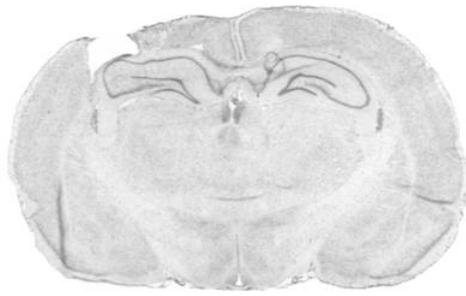


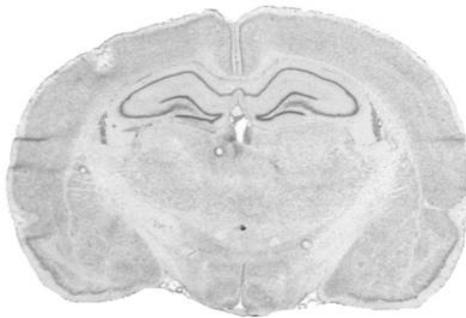
Figure 3.10. Cortical tissue sparing analysis was performed on nissl stained sections. The area of intact cortex was measured on each side of the brain separately using nine equally spaced brain sections. The percentage of cortical tissue spared was calculated by dividing the mean cortical area for the ipsilateral hemisphere by the mean cortical area for the contralateral side of the brain, multiplied by 100. Individual data are shown for each group; bars represent the group mean. Abbreviation: GAL, galantamine



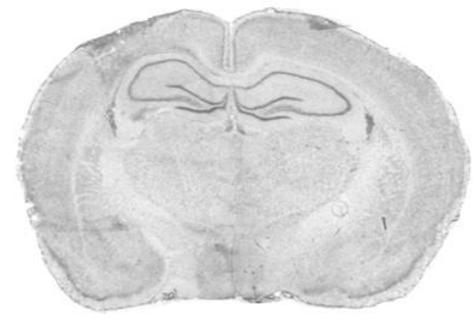
Saline Days 1-11



Galantamine Days 1-2



Galantamine Days 2-6



Galantamine Days 7-11

Figure 3.11. Representative nissl stained sections are shown from the cortical sparing analysis (Figure 3.10) evaluating galantamine treatment following TBI. The injured cortex is shown on the left.

Chapter 4: Experimental Traumatic Brain Injury Reduces the Expression of Hippocampal $\alpha 7$ nAChR mRNA

Introduction

Each year in the U.S., about 1.4 million individuals suffer from traumatic brain injury (TBI) (Langlois et al., 2006), and approximately 5.3 million Americans are living with long term functional changes in thinking, sensation, language, and emotion as a result of TBI (Thurman et al., 1999; NINDS, 2002). Many patients experience neurobehavioral sequelae following TBI characterized by cognitive impairment involving deficits in arousal, attention, memory, and executive functioning (Rao and Lyketsos, 2000; Arciniegas, 2003). Studies suggest that such long term changes significantly impact quality of life and may occur in mild TBI as well as more severe forms of injury (Ashman et al., 2006; Silver et al., 2009). TBI induced alterations in brain neurotransmitter systems could contribute to some of the changes in cognitive and behavioral function following TBI.

Clinical investigations have demonstrated a disruption in cholinergic neurotransmission following TBI. In a clinical study, imaging and neuropsychological examinations linked the cognitive sequelae of TBI to the basal forebrain (Salmond et al., 2005). Postmortem brain studies of individuals with fatal head injuries showed deficits in cortical and cingulate gyrus choline acetyltransferase activity (ChAT) and cingulate synaptophysin immunoreactivity, a measure of synapses, both indicative of presynaptic cholinergic deficits following TBI (Murdoch et al., 1998). In addition, cortical cholinergic innervation was disrupted due to damage of the nucleus basalis of Meynert (Murdoch et al., 2002). Compared to control brains, however, [3 H]-nicotine binding sites, were unaltered (Murdoch et al., 1998). Another report showed temporal cortical ChAT activity decreased by 50 percent in the postmortem brain of patients with fatal head injuries, but M1 and M2 muscarinic acetylcholine (mAChR) receptor binding was unaltered (Dewar and Graham, 1996).

In experimental traumatic brain injury, vesicular acetylcholine transporter immunostaining was enhanced in the hippocampus and cortex, and M2 mAChR

immunoreactivity was decreased in the hippocampus one year following a controlled cortical impact (CCI) (Dixon et al., 1999). Further studies showed this effect as early as two weeks in the hippocampus (Ciallella et al., 1998) with no change in mRNA (Shao et al., 1999). Using an alternative model of TBI, fluid percussion resulted in memory deficits and decreases in ChAT activity in the dorsal hippocampus and frontal and temporal cortices one hour post injury. However, this was accompanied by an increase in ChAT activity in the parietal cortex and a delayed increase in the medial septal area (Gorman et al., 1996). Fluid percussion injury also caused a decrease in hippocampal M2 mAChR binding 24 hours following injury with no change in the M1 mAChR (DeAngelis et al., 1994). In contrast, enhanced binding of total mAChR sites was reported 15 days post injury in the hippocampus and neocortex (Jiang et al., 1994), but no change was found at one or 24 hours following injury, and mAChR affinity was increased at one hour in the hippocampus (Lyeth et al., 1994). In summary, inconsistent changes in presynaptic cholinergic markers and muscarinic receptors have been demonstrated following TBI.

The $\alpha 7$ nicotinic acetylcholine receptor (nAChR) is a homomeric ligand gated ion channel located in hippocampal and cortical regions of the rodent brain, and the high calcium permeability of the $\alpha 7$ nAChR (Seguela et al., 1993) makes it unique among nicotinic receptor subtypes. Although the endogenous functions of $\alpha 7$ receptors are not clearly understood, previous studies have implicated these proteins in processes including learning and memory, synaptic plasticity, neuroprotection, inflammation, and presynaptic regulation of neurotransmitter release (Levin et al., 2002).

Previous studies from our laboratory have shown that experimental traumatic brain injury causes a widespread and significant loss of $\alpha 7$ binding in hippocampal and cortical brain regions (Verbois et al., 2000). $\alpha 7$ receptor downregulation occurs rapidly following a cortical contusion impact (CCI) injury (within one hour), and persists for at least 21 days in some brain regions. Deficits in $\alpha 7$ nAChR expression are dependent on the severity of injury, with more severe damage causing greater downregulation of receptor expression. Forty-eight hours after rats were subjected to a mild CCI, there were significant decreases in multiple cortical and hippocampal brain regions in BTX binding, representing $\alpha 7$ receptor density, in both the injured and uninjured sides of the brain

compared to sham-operated animals. Deficits were found in auditory cortex, CA1, CA2 and CA3 subfields of the hippocampus, dentate gyrus, stratum oriens, subiculum and superior colliculus. In rats receiving a moderate, 2 mm injury, many regions on the contralateral side of the brain showed a significant decrease in $\alpha 7$ nAChR densities compared to sham, but hippocampal BTX binding was even further reduced on the injured side including some subfields demonstrating a 50 percent reduction compared to sham. Conversely, EPI binding, measuring non- $\alpha 7$ nAChRs, was not diminished and was found to be significantly elevated in the auditory cortex in both levels of injury. These results suggest that changes in $\alpha 7$ nAChR expression following TBI could contribute to impaired cognition or neurobehavioral disorders following TBI.

In a time course study, 2 mm injury caused persistent deficits in $\alpha 7$ nAChR binding in the stratum oriens, lateral blade of the dentate gyrus, and CA2 subfield of the hippocampus at one hour following TBI through 21 days, the last time point tested. Hippocampal CA1 deficits occurred at one hour but returned to baseline by the third day. Deficits in the subiculum and cortex occurred by one day but were transient only lasting 72 hours. The CA3 hippocampal subfield and superior colliculus showed deficits by one day and 72 hours, respectively, but continued during the 21 day period of testing. Changes in EPI binding were delayed, some transient and some persistent (Verbois et al., 2002).

Moreover, TBI is associated with cognitive impairment demonstrated in the Morris water maze task of learning and memory, and treatment with nicotine (Verbois et al., 2003b; Verbois et al., 2003a) and the $\alpha 7$ nAChR selective ligand, choline, (Guseva et al., 2008) attenuate this deficit; thus, $\alpha 7$ nAChRs may be important mediators of cell death and survival pathways in the hippocampus and cortex. The results presented in Chapter 3, however, demonstrated an impairment in agonist induced upregulation following TBI. Hence, the purpose of this study was to obtain mechanistic information regarding the plasticity of $\alpha 7$ and non- $\alpha 7$ nAChR expression following TBI. Previous studies have shown that pharmacologically mediated changes in nAChR receptor protein expression (Marks et al., 1992) (Pauly and Collins, unpublished data) occur independent of transcriptional mechanisms. However, transcriptional mechanisms may be involved in TBI induced changes in $\alpha 7$ nAChR expression. We hypothesized that deficits in $\alpha 7$

nAChR expression two days following experimental traumatic brain injury are due to reduced expression of $\alpha 7$ nAChR mRNA. To date, this is the first analysis to employ *in situ* hybridization techniques in order to ascertain possible mechanisms of $\alpha 7$ nAChR downregulation following experimental TBI.

Materials and Methods

Animals and tissue preparation

Rats were housed in a temperature controlled room on a 12 hour light/dark cycle at the University of Kentucky for at least one week prior to surgeries and were allowed unlimited access to food and water. All procedures were done in accordance with the University of Kentucky IACUC guidelines. Nineteen adult male Sprague-Dawley rats were randomly assigned to receive a 1 mm (n = 7) or 2 mm (n = 7) controlled cortical impact (CCI) brain injury or sham craniotomy surgery (n = 5). Each rat was anaesthetized with 4% isoflurane and immobilized in a Kopf stereotaxic frame. A craniotomy (6 mm) was performed using a Michele trephine (Miltex, Lake Success, NY) midway between bregma and lambda above the somatosensory cortex (bregma -2.8 mm, 2.5 mm lateral), and the skull cap was carefully removed. An electronically controlled nitrogen driven piston (TBI-0300 Impactor, Precision Systems and Instrumentation, LLC) was then placed on the surface of the exposed brain and used to administer a 1 mm (mild) or 2 mm (moderate) cortical deformation 5 mm in diameter at a target velocity of 3.5 m/s. Sham animals underwent identical procedures without impaction. After the skull cap was replaced, surgiseal (Johnson & Johnson, Arlington, TX), dental acrylic, and staples were applied and rats were placed back in their cages. Surgeries and instruments are described in detail elsewhere (Scheff et al., 1997; Scheff and Sullivan, 1999). Animals were euthanatized 48 hours following surgery and brains were excised, frozen in isopentane, and sectioned 16 microns thick using a Leica CM50 cryostat (Nussloch, Germany). Tissues were then mounted onto slides coated with gelatin, chromium potassium sulfate and poly-L-lysine to promote tissue adherence. Serial sets of slides were collected throughout the entire rostro-caudal axis of the rat brain, beginning at approximately plate 15 (bregma 0.70 mm) and ending at approximately plate 44 (bregma -6.72 mm) according to Paxinos and Watson's rat brain atlas (Paxinos and Watson,

1986). Sections were made for mRNA and protein studies and were stored at -80°C until use at which time they were thawed and air dried. All reagents were purchased from either Sigma-Aldrich (St. Louis, MO) or Fisher-Scientific (Pittsburgh, PA) unless otherwise stated.

Nicotinic receptor autoradiography

Receptor autoradiography was performed to investigate nicotinic receptor binding using the radioligands, α -[¹²⁵I]-bungarotoxin and [¹²⁵I]-epibatidine, selective for the binding of α 7 nAChRs and non- α 7 nAChRs, respectively. All radioligands were purchased from PerkinElmer Life Sciences, Inc., Boston, MA. α -[¹²⁵I]-bungarotoxin (BTX) binding was carried out as previously described by Pauly and Collins (1993). The concentration of radioligand used was 2-3 fold greater than the affinity (K_d) for binding to the receptor to ensure the assay results are representative of changes in receptor number (B_{max}) as opposed to alterations in affinity. In detail, brain sections were first pre-incubated in Krebs-Ringer HEPES (KRH) buffer, pH 7.5 (1180 mM NaCl, 48 mM KCl, 25 mM CaCl₂, 12 mM MgSO₄(7H₂O), 200 mM HEPES, and 100 mM NaOH) for 30 minutes at room temperature. Next, 2.5 nM α -[¹²⁵I]-BTX (specific activity = 84.0 Ci/mmol) was added to KRH buffer containing 0.5 mg/ml bovine serum albumin, to protect the radioligand from proteases and limit adherence to plastic or glass, in which sections were incubated for two hours at room temperature. This was followed by a series of washes (three x 20 minutes in KRH, one x 10 seconds in 10% KRH, and one x 10 seconds in double deionized water [ddH₂O]) at 4°C.

Non- α 7 nAChR binding was determined through the use of [¹²⁵I]-epibatidine (EPI), specific activity = 2200 Ci/mmol, according to the method of Perry and Kellar (Perry and Kellar, 1995). Optimally, 800 pM of tritiated EPI is employed for the detection of non- α 7 nAChR binding, but due to the unavailability of film at the time of experimentation, the iodinated radioisotope was used instead. [¹²⁵I]-EPI possesses similar properties to the tritiated form such as a high affinity and low non-specific binding (Davila-Garcia et al., 1997; Whiteaker et al., 2000). However, due to a significantly higher specific activity of the iodinated form and limited resources, it was not possible to purchase the radioligand in sufficient concentration to conduct assays at

saturation. Therefore, 1 nM was obtained by adding a sufficient amount of cold EPI (5 mg vial, FW 281.6; adding amount of EPI needed to increase hot EPI amount to 1 nM). Brain sections were incubated with EPI in a pH 7.0 buffer containing 50 mM Tris HCl, 120 mM NaCl, 5 mM MgCl₂, and 2.5 mM CaCl₂ for 40 minutes at room temperature. The incubation was followed by a number of washes at 4°C (two x 5 minutes in buffer, one x 10 seconds in 10% buffer, and one x 10 seconds in ddH₂O).

Once the washes were completed, sections were dried under a low speed fan and stored overnight under vacuum in a desiccator at room temperature. The following day, sections from the BTX and EPI binding assays were exposed to Kodak Biomax MR film. Exposures were stored in x-ray cassettes for eight days or three weeks for BTX and EPI binding assays, respectively. Films were processed with Kodak GBX developer and Photoflo 200 solution (Eastman Kodak Co., Rochester, NY).

Nicotinic receptor mRNA in situ hybridization.

In situ RNA hybridization was performed using riboprobes as previously described and validated (Marks et al., 1992; Marks et al., 1996) and is a modification of earlier published procedures (Simmons et al., 1989; Wada et al., 1989). α -[³⁵S]-radiolabeled-cRNA probes specific for the α 7 and α 4 nicotinic receptor subunits, respectively, were used to study nAChR mRNA. All procedures were performed with collaborators located at the University of Colorado, Boulder. Using α -[³⁵S]UTP (1.0 mCi, Perkin Elmer NEG-039C), probes were made using *in vitro* transcription. For α 7 mRNA, a cRNA probe was prepared using the HIP306s construct (approximately 500 base pairs at the 5'-end of the rat α 7 gene) kindly provided originally by Dr. Jim Boulter of UCLA, Los Angeles, CA. The construct was cloned in Bluescript, the plasmid was linearized with *Eco*RI, and the cRNA was synthesized with T3 RNA polymerase. Constructs for probe synthesis for α 4 mRNA included: clone pHYA-23-1E(2), cloned in pSP64 (also originally provided by Dr. Boulter), linearized with *Eco*RI (Promega, Madison, WI), and synthesized using SP6 RNA polymerase (Promega, Madison, WI). The cRNA was stored at -20°C as a precipitate in 70% ethanol.

In detail, for the [³⁵S]-RNA probe synthesis: 150 μ Ci of [³⁵S]-UTP was added to a sterile 1.5 ml eppendorf tube and evaporated to dryness. Next the following were

added with a vortex after each addition: 1 μ l 5X reaction buffer (Promega, Madison, WI), 0.5 μ l 1M dithiothreitol (DTT, a reducing agent to help protect the label), 1 μ l adenosine triphosphate, cytosine triphosphate, and guanosine triphosphate (CTP/ATP/GTP mixture, Boehringer-Mannheim, Indianapolis, IN), and 1 μ l linearized template. Then 0.5 μ l RNaseIN and 1 μ l SP6 RNA polymerase were each added followed by gentle mixing after each addition. Next the tube was incubated at 37°C for at least one hour. A DNA hydrolysis step followed: 25 μ l of 50 mM Tris/10 mM MgCl₂ was added with a vortex and 0.75 μ l RQ DNase and 0.25 μ l RNaseIN (Promega, Madison, WI) were added with a gentle mix after each. The tube was then incubated at 37°C for 30 minutes. The last step involves precipitation: 2.5 μ l 10 mg/ml tRNA, 10 μ l 10 M NH₄OAc, and 80 μ l absolute ETOH were added with a vortex after each followed by incubation at -20°C for one hour. Next the mixture was centrifuged for 10 minutes at about 25,000 rpm in a cold room and the supernatant was removed. The pellet was then resuspended in 50 μ l diethylpyrocarbonate (DEPC) treated water (1 ml/L ddH₂O incubated for one hour at 37°C and then autoclaved) and 1 μ l of 1 M DTT was added followed by a vortex. Cpm/ μ l was determined by counting the probe solution.

For the following hybridization procedures, up to the ribonuclease (RNase) treatment step, glassware was baked at 200°C for two hours and RNase free equipment and utensils were used. All reagents were made using DEPC treated water. To begin the hybridization procedures, slides were removed from the freezer, let warm to room temperature under vacuum and sections were pulse fixed for 15 minutes with 4% paraformaldehyde in phosphate buffered saline (PBS: 137 mM NaCl, 2.5 mM KCl, 16 mM, Na₂HPO₄, and 4 mM NaH₂PO₄, pH = 7.4) and washed three times for five minutes each in PBS and then air dried.

Next sections were acetylated by incubation in 15 mM acetic anhydride and 0.1M triethanolamine, pH = 8.0, for 10 minutes and rinsed for two minutes in 2X standard saline citrate (SSC, 1X SSC: 150 mM NaCl, 15 mM trisodium citrate, pH to 7.0 with HCl) and dehydrated with a series of graded ETOH solutions (three minutes each: 50, 70, 95, 100 and 100%). Samples were air dried and stored under vacuum in a desiccator for at least two hours to remove all ETOH for hybridizations that same day.

For the $\alpha 4$ probe only, a hydrolysis procedure was performed using the method of Cox et al. (1984) to provide an average probe size of approximately 500 bases. The cRNA probe was placed in a 60°C water bath with the addition of 50 μ l carbonate mix (83mM NaHCO₃, 180 mM Na₂CO₃, q.s. 5 ml DEPC water) for 15 minutes. The reaction was stopped by adding 8 μ l of acetate stop (1.15 M NaOH, 2.2M HOAc, q.s. 10 ml DEPC water) followed by 220 μ l absolute ethanol with an incubation of 30 minutes at -20°C. Next the tube was centrifuged for five minutes and the supernatant removed followed by resuspension in 100 μ l DEPC water.

For the hybridization step, [³⁵S]-radiolabeled cRNA probes were dissolved in hybridization buffer (50% formamide, 10% dextran sulfate, 300 mM NaCl, 10 mM Tris, 1 mM ethylenediamine tetra acetate [EDTA], 500 μ g/ml yeast tRNA, 10 mM DTT, 1X Denhardt's solution, pH = 8.0) with a final concentration of 5 x 10⁶ cpm/ml in DEPC. Next the solution was vortexed and incubated for 10 minutes at 60°C. To initiate the hybridization, the solution was added to a glass microscope slide coverslip (24 mm x 60 mm) and this was attached to the samples by turning the slides containing tissue sections face down. The microscope slide and coverslip edges were sealed using DPX (BDH, Poole, England), and tissues were incubated at 58°C in a dry oven for 12-18 hours.

Finally, a wash protocol was performed in which all water used was ddH₂O, not DEPC treated water. First, DPX was removed and coverslips were prepared for removal by washing in 4X SSC for 15 minutes with agitation. Then the slides were washed four more times for five minutes each in 4X SSC. Then samples were incubated in a ribonuclease-containing buffer (20 μ g/ml RNaseA, 500 mM NaCl, 10 mM Tris [pH = 8.0], 1 mM EDTA) at 37°C for 30 minutes to digest non-hybridized, single-stranded RNA. Sections were washed and desalted by incubation (5 minutes each) in 2X SSC three sequential times, then 1X SSC, and 0.5X SSC (all containing 1 mM DTT to prevent oxidation). This was followed by a 30 minute high stringency wash with incubation in 0.1X SSC plus 1 mM DTT at 60°C. Samples were then transferred to an identical SSC, DTT solution and cooled for 10 minutes and then dehydrated by the ethanol series described earlier. The slides were air dried and exposed to Kodak Biomax MR film for 21 days or 12 hours for $\alpha 7$ and $\alpha 4$ mRNA, respectively, and developed as described for the receptor autoradiography studies.

Image analysis and statistics

All binding and hybridization data were analyzed using NIH image v1.59 on a Power Macintosh connected to a Sony XC-77 CCD camera via a Scion LG-3 frame-grabber. Nicotinic receptor binding determinations and *in situ* hybridization data were obtained as uncalibrated optical density measurements. Statistical analysis was performed using a two-way (injury, side), repeated measures, analysis of variance (ANOVA) followed by a Tukey-Kramer multiple comparisons test (GBSTAT software) for the autoradiography studies. Due to apparent variations in the degree of $\alpha 4$ probe hybridization within each microscope slide and between films, each brain slice was used as its own control. Data from the nAChR *in situ* hybridization studies were analyzed as the calculated percent change in optical density from control, (ipsilateral/contralateral)*100, for each rat using a one-way ANOVA followed by a Tukey-Kramer multiple comparison test. For consistency, both $\alpha 7$ and $\alpha 4$ mRNA data were analyzed and presented herein using this method. In addition, the $\alpha 7$ nAChR mRNA ipsilateral and contralateral measurements and analysis can be found in the appendix, as this data was used for a correlation analysis. Lastly, Pearson correlation analysis was employed to examine the relationship between $\alpha 7$ nAChR density and $\alpha 7$ mRNA using GraphPad Prism version 5.01 (San Diego, CA). Significance was set at $\alpha = 0.05$ for all statistical procedures.

Results

Receptor autoradiography was performed in order to measure $\alpha 7$ nAChR density in rats undergoing sham surgery, 1 mm CCI or 2 mm CCI. The BTX binding analysis in multiple brain areas including cortical, thalamic, and hippocampal regions is presented in Table 4.1. Due to the multiplicity of repeated measures comparisons and difficulty of interpretation thereof, only significant results directly comparing the ipsilateral and contralateral side of the brain within the same level of injury (side effect) and significant results comparing the ipsilateral side of the brain between the three injury groups (injury effect) will be presented. For all studies conducted herein, when comparisons between the ipsilateral and contralateral sides of sham operated rats were made, no significant differences were detected.

In rats subjected to a 1 mm CCI, BTX binding was significantly increased in the ipsilateral auditory cortex layers 1-4 compared to the contralateral side, $F(1,16) = 8.81, p = 0.0091$. Following a 1 mm CCI, the lateral blade of the dentate gyrus (anterior hippocampus) displayed reduced BTX binding ipsilaterally compared to sham, $F(2,16) = 15.82, p = 0.0002$.

In rats that received a 2 mm CCI, BTX binding was significantly decreased in the ipsilateral stratum oriens, $F(1,16) = 26.56, p < 0.0001$, hilar blade of the dentate gyrus, $F(1,16) = 12.33, p = 0.0029$, ventrolateral geniculate nucleus, $F(1,16) = 11.26, p = 0.004$, and superior colliculus, $F(1,16) = 12.82, p = 0.0025$, compared to the contralateral side. Following 2 mm CCI, there was also a significant reduction in the ipsilateral auditory cortex layers 1-4, $F(2,16) = 8.27, p = 0.0034$, auditory cortex layers 5-6, $F(2,16) = 7.57, p = 0.0048$, stratum oriens, $F(2,16) = 4.67, p = 0.0253$, lateral blade of the dentate gyrus (anterior hippocampus), $F(2,16) = 15.82, p = 0.0002$, and superior colliculus, $F(2,16) = 6.13, p = 0.0106$, compared to the ipsilateral side of the brain in 1 mm CCI and sham operated rats.

Lastly, a non-significant trend toward a reduction was detected following 2 mm CCI in the ipsilateral hilar blade measured at the level of the posterior hippocampus compared to the ipsilateral side in 1 mm CCI and sham groups, $F(2,16) = 3.62, p = 0.0504$ (borderline ANOVA, Tukey-Kramer, $p < 0.01$).

In situ hybridization followed by semi-quantitative autoradiographic analysis was performed to detect $\alpha 7$ nAChR mRNA levels in rats undergoing sham surgery, 1 mm CCI or 2 mm CCI. Table 4.2 shows $\alpha 7$ nAChR mRNA expression in multiple areas throughout the brain including several hippocampal and cortical regions. Group mean data are obtained from calculating the percent change from control as follows: ipsilateral optical density divided by the contralateral optical density, multiplied by 100 for each rat. Data are expressed in this manner in order to be consistent with the $\alpha 4$ data in which technical issues with the experiment were noted and warranted this approach. For completeness and comparison with the BTX binding data, the ipsilateral and contralateral data are presented in Appendix I.

Following a 1 mm CCI, there was a significant reduction in $\alpha 7$ mRNA only in the lateral blade of the dentate gyrus, $F(2, 16) = 11.95, p = 0.0007$, compared to sham

operated rats. This reduction was also present in this brain region following a 2 mm CCI. Expression of $\alpha 7$ mRNA following 2 mm CCI was also significantly reduced in the CA2 layer of the hippocampus, $F(2, 16) = 15.79, p = 0.0002$, the CA3 layer of the hippocampus, $F(2, 16) = 11.38, p = 0.0008$, and the hilar blade of the dentate gyrus, $F(2, 16) = 18.66, p < 0.0001$. Significant reductions were found when compared to both sham and 1 mm CCI rats. Furthermore, there was a significant downregulation of $\alpha 7$ mRNA in the superior colliculus following 2 mm CCI compared to sham operated rats $F(2, 16) = 7.22, p = 0.0058$. By contrast, a slight elevation in $\alpha 7$ mRNA was noted in the inner layers of the auditory cortex (layers 5-6) in the 2 mm CCI group; however, this upregulation was not statistically significant. Deficits in $\alpha 7$ mRNA expression correlate well with the reductions in $\alpha 7$ nAChR binding obtained from previous studies by our group and the study herein as well as support the literature on cholinergic neurotransmission alterations in TBI.

Figure 4.1 includes representative autoradiographs demonstrating reductions in hippocampal regions in both BTX binding and $\alpha 7$ mRNA expression.

The results of the EPI binding analysis measuring non- $\alpha 7$ nAChR receptor density are presented in Table 4.3 in which several cortical regions and thalamic nuclei were examined. In order to simplify complex comparisons, only significant results directly comparing the ipsilateral and contralateral side within an injury treatment group and significant results comparing the ipsilateral side of the brain among the three injury groups will be presented.

There were no significant changes in EPI binding following a 1 mm CCI. However, EPI binding was significantly reduced following 2 mm CCI on the ipsilateral side of the brain compared to the contralateral side of the brain in auditory cortex layers 4-6, $F(1, 16) = 4.80, p = 0.0437$, and auditory cortex layers 2-3, $F(1, 16) = 24.16, p = 0.0002$. The ipsilateral auditory cortex layers 2-3 was also reduced when compared to the injured side of the brain in 1 mm CCI and sham operated rats $F(2, 16) = 3.94, p = 0.0407$. In the anterodorsal thalamic nucleus/dorsal geniculate nucleus, EPI was significantly decreased on the ipsilateral side of the brain in rats receiving a 2 mm CCI compared to the contralateral side of the brain $F(1, 16) = 6.20, p = 0.0242$. Of note, the ipsilateral side of 2 mm CCI rats showed the lowest mean for all regions except for the ventrolateral

geniculate and medial geniculate nuclei. These regions were difficult to measure, in particular the medial geniculate, because in several cases, multiple sections containing this region were not available.

The $\alpha 4$ nAChR *in situ* hybridization data are presented in Table 4.4. Interestingly, 2 mm CCI significantly reduced $\alpha 4$ nAChR mRNA in the thalamus, $F(2,16) = 7.70$, $p = 0.0045$, and dorsal geniculate nucleus, $F(2,16) = 25.00$, $p < 0.0001$, compared to 1 mm CCI treated rats and sham operated rats. In addition, $\alpha 4$ nAChR mRNA was significantly reduced following 2 mm CCI in the medial geniculate nucleus, $F(2,16) = 6.27$, $p = 0.0098$, compared to 1 mm CCI treated rats. Because the contralateral measurement for each brain section was used as a control to generate individual rat brain optical density measurements, ANOVA was used to verify the measurements were not affected by the CCI. ANOVA of the contralateral optical density means revealed no significant differences between groups. Figure 4.2 includes representative autoradiographs demonstrating $\alpha 4$ mRNA and EPI binding in thalamic nuclei.

Correlation analysis was performed to further describe the results of the BTX and $\alpha 7$ mRNA analyses in rats following TBI. Pearson correlation was used to determine the relationship between $\alpha 7$ nAChR density and $\alpha 7$ mRNA in ipsilateral brain regions previously determined in the current study to be significantly altered 48 hours following TBI. These regions include auditory cortical layers 1-4 and 5-6, hippocampal subfields CA1 and CA3, the lateral and hilar blades of the dentate gyrus, and the superior colliculus (Figure 4.3). Two of the seven correlations were statistically significant. The $\alpha 7$ nAChR density and mRNA were significantly positively correlated in the auditory cortex layers 1-4 ($r=0.4911$, $p=0.0328$) and the lateral blade of the dentate gyrus ($r=0.6001$, $p=0.006$). Contrary to all other relationships shown, the superior colliculus demonstrated an inverse relationship; as the $\alpha 7$ nAChR density increased, the $\alpha 7$ mRNA is downregulated. However, this relationship did not reach statistical significance.

Discussion

Previously our laboratory demonstrated significant reductions in hippocampal and cortical $\alpha 7$ nicotinic receptor binding in rats subjected to CCI. In the current study, *in*

situ hybridization was performed to investigate one possible mechanism involved in $\alpha 7$ nAChR expression deficits following traumatic brain injury. The results of this study are the first to show downregulation of $\alpha 7$ mRNA accompanying reductions in $\alpha 7$ nAChR binding in rats 48 hours following CCI. Multiple regions throughout the brain were analyzed, with emphasis on cortical and hippocampal regions important in cognition and memory.

A summary of the results from the nAChR determinations displays the number of ipsilateral brain regions significantly altered following 1 or 2 mm CCI (Table 4.5). TBI caused a significant reduction in $\alpha 7$ mRNA in the lateral and hilar blades the dentate gyrus, the CA2 and CA3 subfields of the hippocampus, and the superior colliculus, which is a midbrain nucleus involved in visual processing.

Analysis of $\alpha 7$ nAChR density as measured by BTX binding correlated well with our laboratory's previous studies (Verbois et al., 2000). Saturation analysis of BTX binding in an identical experimental paradigm of TBI showed results were attributed to changes in binding sites or receptor density and not affinity for the receptor (Verbois et al., 2002). In the current study, TBI resulted in decreases in BTX binding in regions of the hippocampus, thalamus, and brain stem. Importantly, nicotinic receptor mRNA is present mostly in the cell body of neurons and nAChRs are located predominantly on presynaptic nerve terminals. Thus, there may be some discrepancies between regions analyzed for mRNA measurements and those analyzed for receptor densities.

A correlation analysis was performed to further characterize the relationship between BTX binding and $\alpha 7$ mRNA. The auditory cortex layers 1-4 and the lateral blade of the dentate gyrus showed significant correlations which helps to confirm that the changes in the expression of $\alpha 7$ nAChR are due to transcriptional mechanisms in these regions. A non-significant inverse correlation was found in the superior colliculus, which may suggest that nAChRs are regulated differently based on cell type and location. Depending on cell type, the presence of cellular mediators have been shown to regulate transcription and subsequent cell surface receptor assembly differently (Albuquerque et al., 2009). Transcriptional factors such as the upstream stimulatory factor 1 (USF-1), early growth response 1 (Egr-1), specificity protein 1 (Sp1), and specificity protein 3 (Sp3) have been shown to regulate the rat $\alpha 7$ promoter (Nagavarapu et al., 2001). It is

not known how neurochemical changes following TBI might affect the $\alpha 7$ promoter and the transcription of the nAChR.

In contrast to the downregulation in nAChRs, rats displayed elevated $\alpha 7$ mRNA in the inner layers (layers 5-6) of the auditory cortex two days following TBI. There was also a non-significant trend toward an increase in BTX binding following a 1 mm CCI (Figure 5.1). These results are further evidence that nAChRs may be regulated differently based on location. It is possible that regional differences reflect compensatory changes. It is also possible that the presence of pathophysiological mediators including APP or A β could contribute to these alterations.

In this model of TBI, the CCI does not physically penetrate the hippocampus, but studies employing this method of experimental injury show that there is cell death and increased neuroinflammation in portions of the rat hippocampus following CCI (Scheff et al., 1997). Rats subjected to a 1 mm CCI had no or mild damage to the hilus and CA3 subfield of the hippocampus and rats undergoing a 2 mm CCI had mostly severe damage to the aforementioned subfields at least seven days after injury. Further analysis by this group using an optical dissector method showed that ipsilateral CA3 cell loss occurred as early as one hour following injury and decreased to 41% of control by one day after injury (Baldwin et al., 1997). While the neuronal loss affects the interpretation of the binding results in the hilus and hippocampal subfield CA3 at the most severe injury level, the deficits in $\alpha 7$ mRNA and $\alpha 7$ nAChR are widespread and other receptor subtypes such as mAChR (Verbois et al., 2000) do not appear to be as sensitive to the effects of TBI.

Changes in non- $\alpha 7$ nAChR expression were also evaluated following TBI in order to determine if diminished nicotinic receptor binding following TBI is selective to the $\alpha 7$ receptor. Therefore, $\alpha 4$ mRNA was evaluated by *in situ* hybridization and non- $\alpha 7$ nAChRs were measured by autoradiography with EPI, which binds to non- $\alpha 7$ nAChRs (the most abundant being the $\alpha 4\beta 2$ subtype). $\alpha 4$ mRNA was not detectable in the hippocampal formation, but instead was measured in regions of the diencephalon, rhinencephalon and mesencephalon, in particular cortical and thalamic nuclei. Likewise, EPI binding was minimal in the hippocampal formation except for a low level of expression in the lateral blade of the dentate gyrus. Following TBI, $\alpha 4$ mRNA was significantly reduced in the thalamus and two regions of the metathalamus, the dorsal

geniculate and medial geniculate nuclei. The reduction of $\alpha 4$ mRNA in brain regions of the diencephalon was also seen in EPI binding, for EPI binding was decreased in the dorsal geniculate nucleus. In addition, contrary to previous studies from our laboratory that showed upregulation in the auditory cortex, EPI binding was also diminished in the auditory cortex, which was not detected in $\alpha 4$ mRNA. Of note, measurements of the auditory cortex were obtained including all layers for mRNA analysis instead of separating layers as was done for EPI analysis. Furthermore, the contribution of the $\beta 2$ subunit which generally is co-localized with $\alpha 4$ containing receptors may explain this difference. $\beta 2$ mRNA was not measured in the present study and would be useful in future studies to enhance the understanding of nicotinic receptor involvement in TBI.

The cellular mechanisms that regulate expression of nicotinic receptor subtypes are still largely unknown. Chronic treatment with nAChR agonists or antagonists is well known to cause an increase in the density of both $\alpha 7$ and non- $\alpha 7$ nAChR subtypes. Mice chronically treated with nicotine for 10 days displayed significant increases in nicotinic receptor binding in many regions throughout the brain, as measured by [3 H]-nicotine binding (Marks et al., 1983; Marks et al., 1985; Marks et al., 1992; Robinson et al., 1996) and to a lesser extent by α -[125 I]-BTX (Marks et al., 1983; Pauly et al., 1991), but this did not correlate with a significant upregulation in $\alpha 4$ or $\beta 2$ subunit mRNA detected by *in situ* hybridization (Marks et al., 1992). Furthermore, rats infused with nicotine for two weeks demonstrated upregulated [3 H]-methyllycaconitine binding of $\alpha 7$ nAChRs but no change in $\alpha 7$ and $\alpha 6$ mRNA (Mugnaini et al., 2002). As nicotinic receptor up-regulation does not appear to require changes in steady state mRNA expression in these models, this suggests post translational changes in receptor assembly, insertion or turnover may be involved (Gentry and Lukas, 2002; Gaimarri et al., 2007). Additionally, protein tyrosine dephosphorylation may also be involved in nAChR regulation, as inhibition of protein tyrosine kinases has been shown to cause upregulation of receptor density and function (Cho et al., 2005). The results presented in Chapter 3 suggest that TBI impairs nAChR upregulation, and this may be due to the loss of nAChR mRNA following TBI demonstrated in the current study. Lower concentrations of pre-formed RNA pools would prevent levels of upregulation expected with treatment of nAChR agonists.

Nicotinic receptors have also been shown to be regulated by corticosteroids. In contrast to the effects of nicotinic agonists and antagonists, corticosteroid treatment decreases $\alpha 7$ nAChR protein expression in mouse brain. Chronic corticosterone for seven or nine days resulted in a widespread significant decrease in α -[¹²⁵I]-BTX binding (Pauly and Collins, 1993; Robinson et al., 1996), and [³H]-nicotinic binding sites were less sensitive to this effect (Pauly and Collins, 1993). Furthermore, no change in nicotinic mRNA was detected following corticosterone treatment (Pauly and Collins, unpublished data). Conversely, one *in vitro* study reported $\alpha 7$ nAChR expressed in bovine chromaffin cells displayed an activation in transcription following dexamethasone treatment (Carrasco-Serrano and Criado, 2004). And in PC12 cells, nicotine upregulated nAChRs but decreased $\alpha 3$ mRNA and only slightly decreased $\beta 2$ subunit indicating upregulation due to a non-transcriptional mechanism (Madhok et al., 1995). The disparity in results may be due to differences in *in vitro* models compared to physiologically intact, whole animal studies. Due to the reductions in mRNA demonstrated in the current study, enhanced corticosteroid concentrations may not contribute significantly to changes in nAChR expression following TBI.

Recently, peripheral nAChRs have been implicated in an anti-inflammatory pathway in which vagus nerve stimulation activates nAChRs located on macrophages and results in the inhibition of proinflammatory cytokines including tumor necrosis factor α (TNF), interleukin 1 (IL-1), and high mobility group box 1 (HMGB1) (Ulloa, 2005). Furthermore, the $\alpha 7$ nAChR has been shown to be present on microglia and regulates microglial activation and inflammatory response; thus, decreases in nAChR receptor activity could play a role in promoting inflammation and therefore neuronal damage (Wang et al., 2003; Shytle et al., 2004). Moreover, nicotine displays immunosuppressive effects through the $\alpha 7$ nAChR receptor (Yoshikawa et al., 2006). One week following CCI, mice displayed increased inflammation in ipsilateral thalamic nuclei as measured by [³H]-PK11195 binding to activated microglia (Kelso et al., 2006). While the $\alpha 4$ nAChR has not specifically been implicated in the regulation of inflammatory processes, a recent report indicated a possible link between anti-inflammatory signaling pathways and the presence of multiple nicotinic subtypes on monocytes including $\alpha 4$ (Blanchet et al., 2006). It is unknown whether inflammation is involved in the alteration in nAChR

expression in thalamic nuclei demonstrated in the current study, and further research is needed to elucidate the cause and effect relationship between nAChRs and inflammation.

Moreover, modulators of nAChRs have been shown to modify the deleterious effects of inflammation. In mice treated with lipopolysaccharide, galantamine pretreatment reduced serum TNF concentrations and protected mice from the deleterious effects of sepsis, and galantamine mediated suppression of TNF levels did not occur in $\alpha 7$ nAChR knock out mice (Pavlov et al., 2009). Likewise, pretreatment with the $\alpha 7$ nAChR selective ligand, choline, also demonstrated anti-inflammatory effects in LPS treated mice. Choline treated mice demonstrated an increase in survival and reduced TNF and HMGB1 levels (Parrish et al., 2008). Studies in $\alpha 7$ nAChR knock out mice showed the effect was mediated through the $\alpha 7$ nAChR. It is unknown if this peripheral reduction in inflammation will also attenuate central nervous system inflammation to impact neuropathology in neurodegenerative disease. Consequently, deficits in nAChR following TBI may result in a number of pathological mechanisms underlying functional outcome.

In summary, moderate TBI demonstrated a pronounced effect on nAChR density and mRNA expression, whereas mild TBI produced only a minor effect. TBI resulted in a significant reduction in hippocampal $\alpha 7$ nAChR binding that was associated with reduced $\alpha 7$ mRNA expression. Reductions in BTX binding were significantly correlated with diminished $\alpha 7$ mRNA expression in the auditory cortex and dentate gyrus. In addition, decreased $\alpha 4$ nAChR binding in the thalamus was accompanied by decreases in EPI binding. These results suggest that TBI has a direct inhibitory effect on the transcriptional regulation of nAChRs 48 hours following injury. Further research is needed to fully elucidate the role of nAChRs in the pathogenesis and treatment of the cognitive dysfunction associated with TBI.

Table 4.1. Alterations in $\alpha 7$ nAChR density, as measured by α -[125 I]-bungarotoxin (BTX) binding, throughout the brain following TBI in rats. Data are expressed as group mean \pm standard deviation optical density.

Brain Region	Sham		1 mm CCI		2 mm CCI	
	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral
Neocortex						
Auditory cortex layers 1-4	56.16 \pm 4.47	57.84 \pm 4.40	51.79 \pm 6.83	59.41 \pm 3.47*	48.18 \pm 4.95	47.76 \pm 3.03† Ψ
Auditory cortex layers 5-6	70.91 \pm 5.51	73.34 \pm 4.09	68.82 \pm 4.93	72.58 \pm 5.11	62.07 \pm 5.31	61.69 \pm 6.27† Ψ
Basal ganglia						
Endopiriform	97.93 \pm 4.92	96.53 \pm 11.04	94.25 \pm 5.74	96.27 \pm 7.15	94.97 \pm 5.37	81.28 \pm 14.36
Diencephalon						
Ventrolateral geniculate n.	109.81 \pm 1.96	109.55 \pm 2.38	109.74 \pm 4.78	106.87 \pm 3.05	109.33 \pm 6.26	101.27 \pm 6.35*
Subthalamic nucleus	122.83 \pm 9.17	126.42 \pm 10.33	123.55 \pm 2.80	123.47 \pm 4.94	121.18 \pm 7.73	122.53 \pm 6.50
Posterior hypothalamic area	110.53 \pm 7.50	108.07 \pm 5.14	108.25 \pm 4.21	108.10 \pm 5.20	110.14 \pm 4.54	106.08 \pm 4.89
Anterior hippocampus						
Stratum Oriens	72.81 \pm 4.72	73.22 \pm 2.95	73.00 \pm 6.23	70.26 \pm 7.67	70.80 \pm 4.15	59.13 \pm 3.94*† Ψ
CA1 layer	47.10 \pm 3.90	48.02 \pm 4.60	44.18 \pm 3.88	45.43 \pm 5.14	43.37 \pm 3.13	45.04 \pm 2.37
CA3 layer	65.08 \pm 5.65	67.84 \pm 3.67	64.81 \pm 6.01	66.55 \pm 5.41	63.03 \pm 3.45	61.73 \pm 2.56
Dentate gyrus, lateral blade	60.77 \pm 3.57	62.41 \pm 4.87	57.40 \pm 4.71	56.30 \pm 4.10†	50.46 \pm 4.45	47.85 \pm 2.15† Ψ
Dentate gyrus, hilar blade	106.67 \pm 3.39	105.65 \pm 2.73	109.36 \pm 8.37	109.70 \pm 10.56	104.78 \pm 6.25	94.64 \pm 7.11*
Posterior hippocampus						
CA3 layer/dentate gyrus	82.34 \pm 2.98	86.41 \pm 3.08	79.01 \pm 6.61	81.46 \pm 5.92	77.71 \pm 7.30	78.70 \pm 7.55
Dentate gyrus, hilar blade	122.51 \pm 11.05	124.36 \pm 9.45	123.99 \pm 8.83	123.16 \pm 10.66	117.78 \pm 5.66	105.74 \pm 11.83
Amygdala						
Medial amygdale	121.24 \pm 9.04	119.88 \pm 15.33	119.09 \pm 6.10	115.61 \pm 5.69	118.57 \pm 9.18	111.21 \pm 14.57
Mesencephalon						
Superior colliculus	128.23 \pm 3.49	128.08 \pm 4.25	127.99 \pm 4.69	125.23 \pm 3.86	122.72 \pm 6.62	117.20 \pm 4.02*† Ψ

* denotes significantly different from the contralateral side

† denotes significantly different from the ipsilateral side of sham operated rats

Ψ denotes significantly different from the ipsilateral side of 1 mm CCI rats

Statistical significance: $\alpha = 0.05$

Abbreviation: n, nucleus

Table 4.2. Changes in $\alpha 7$ nAChR mRNA following TBI in rats measured by *in situ* hybridization. Data are expressed as group mean \pm standard deviation optical density; mean data are obtained from the calculated percent change from control, (ipsilateral/contralateral)*100, optical density for each rat.

Brain Region	Sham % Control	1 mm CCI % Control	2 mm CCI % Control
Neocortex			
Auditory cortex layers 1-4	99.70 \pm 5.07	100.84 \pm 3.68	98.21 \pm 6.31
Auditory cortex layers 5-6	103.88 \pm 3.86	103.56 \pm 3.03	107.87 \pm 5.51
Basal ganglia			
Endopiriform	104.37 \pm 6.84	100.68 \pm 7.46	103.38 \pm 6.72
Anterior hippocampus			
CA1 layer	98.70 \pm 2.77	96.11 \pm 4.83	93.53 \pm 4.47
CA2 layer	102.45 \pm 1.82	100.95 \pm 1.52	96.27 \pm 2.55 $\dagger\Psi$
CA3 layer	101.07 \pm 5.36	98.90 \pm 4.89	84.84 \pm 8.63 $\dagger\Psi$
Dentate gyrus, lateral blade	98.07 \pm 2.30	91.00 \pm 2.62 \dagger	86.75 \pm 5.60 \dagger
Dentate gyrus, hilar blade	96.78 \pm 2.20	94.75 \pm 3.77	84.15 \pm 4.98 $\dagger\Psi$
Posterior hippocampus			
Dentate gyrus, hilar blade	100.02 \pm 0.82	100.09 \pm 4.46	97.68 \pm 4.77
Mesencephalon			
Superior colliculus	100.54 \pm 2.08	98.57 \pm 1.85	96.59 \pm 1.48 \dagger

\dagger denotes significantly different from sham operated rats

Ψ denotes significantly different from 1 mm CCI rats

Statistical significance: $\alpha = 0.05$

Table 4.3. Reductions in non- $\alpha 7$ nAChR expression, measured by [125 I]-epibatidine (EPI) binding, following TBI in rats. Data are expressed as group mean \pm standard deviation optical density.

Brain Region	Sham		1 mm CCI		2 mm CCI	
	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral
Neocortex						
Auditory cortex layers 2-3	54.67 \pm 3.33	56.29 \pm 5.86	55.23 \pm 6.68	54.54 \pm 5.47	54.98 \pm 3.46	42.84 \pm 2.86* \dagger Ψ
Auditory cortex layers 4-6	45.02 \pm 3.42	47.34 \pm 5.06	44.52 \pm 5.87	45.01 \pm 5.43	44.65 \pm 2.80	38.85 \pm 2.55*
Diencephalon						
Thalamus (AV/VL)	100.01 \pm 5.30	100.19 \pm 2.73	98.03 \pm 8.85	99.87 \pm 7.43	97.95 \pm 3.79	92.34 \pm 5.21
Anterodorsal thalamic n./ Dorsal geniculate n.	140.33 \pm 3.31	140.93 \pm 3.64	139.42 \pm 6.04	138.03 \pm 5.49	140.09 \pm 7.45	133.84 \pm 6.62*
Ventrolateral geniculate nucleus	118.47 \pm 10.55	119.12 \pm 14.88	109.34 \pm 8.45	112.47 \pm 9.53	108.86 \pm 6.12	108.78 \pm 8.93
Medial geniculate nucleus	105.43 \pm 22.46	105.61 \pm 24.09	94.57 \pm 8.68	94.83 \pm 10.55	104.55 \pm 16.86	101.24 \pm 14.51

* denotes significantly different from the contralateral side

\dagger denotes significantly different from the ipsilateral side of sham operated rats

Ψ denotes significantly different from the ipsilateral side of 1 mm CCI rats

Statistical significance: $\alpha = 0.05$

Abbreviations: AV, anteroventral thalamic nucleus; VL, ventrolateral thalamic nucleus; n, nucleus

Table 4.4. Diminished levels of thalamic $\alpha 4$ nAChR mRNA as measured by *in situ* hybridization following TBI in rats. Data are expressed as group mean \pm standard deviation optical density; mean data are obtained from the calculated percent change from control, (ipsilateral/contralateral)*100, optical density for each rat.

Brain Region	Sham % Control	1 mm CCI % Control	2 mm CCI % Control
Neocortex			
Auditory cortex	99.03 \pm 6.61	97.44 \pm 7.89	102.00 \pm 16.52
Retrosplenial cortex	99.90 \pm 2.47	95.41 \pm 9.79	89.48 \pm 7.27
Diencephalon			
Anterodorsal thalamic nucleus	102.42 \pm 7.30	90.85 \pm 9.49	70.03 \pm 7.08 $\dagger\Psi$
Thalamus (AV/VL)	100.55 \pm 6.28	96.46 \pm 6.63	86.08 \pm 7.07 $\dagger\Psi$
Ventromedial hypothalamic nucleus	98.58 \pm 2.80	104.81 \pm 9.30	97.72 \pm 6.44
Medial geniculate nucleus	99.27 \pm 5.21	103.29 \pm 14.57	81.77 \pm 12.24 Ψ
Rhinencephalon			
Subiculum	95.79 \pm 3.54	97.17 \pm 19.36	93.20 \pm 10.71
Mesencephalon			
Substantia nigra	104.13 \pm 4.64	104.35 \pm 10.02	98.15 \pm 11.21

\dagger denotes significantly different from sham operated rats

Ψ denotes significantly different from 1 mm CCI rats

Statistical significance: $\alpha = 0.05$

Abbreviations: AV, anteroventral thalamic nucleus; VL, ventrolateral thalamic nucleus

Table 4.5. A summary of the results from the nAChR determinations displays the number of ipsilateral brain regions significantly altered following 1 or 2 mm CCI as presented in the results section. The number of significantly altered regions is expressed as a fraction of the number of brain regions analyzed in each assay.

Assay	Sham		1 mm CCI		2 mm CCI	
	Decrease	Increase	Decrease	Increase	Decrease	Increase
BTX	0/15	0/15	1/15	1/15	7/15	0/15
α7 mRNA	0/10	0/10	1/10	0/10	5/10	0/10
EPI	0/6	0/6	0/6	0/6	3/6	0/6
α4 mRNA	0/8	0/8	0/8	0/8	2/8	0/8

Abbreviations: BTX, α -[¹²⁵I]-bungarotoxin; EPI, [¹²⁵I]-epibatidine

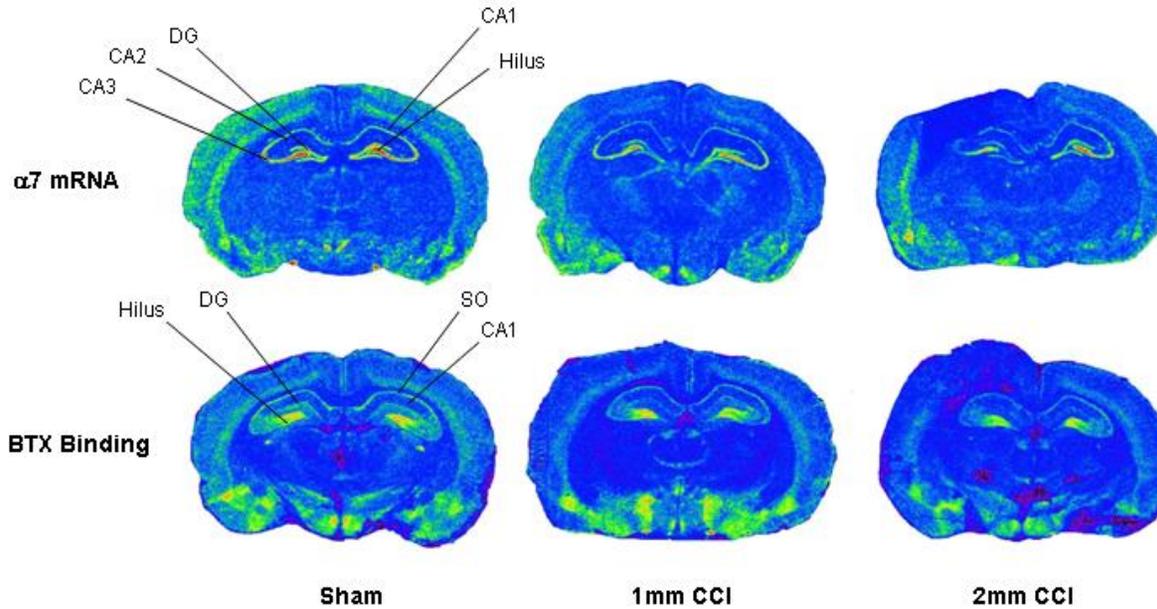


Figure 4.1. $\alpha 7$ nAChR mRNA and protein is reduced in hippocampal regions following TBI. Representative autoradiographs demonstrate $\alpha 7$ mRNA as measured by *in situ* hybridization and $\alpha 7$ nAChR density determined by α -[125 I]-BTX binding. The left side of the brain shown is the ipsilateral side. Abbreviations: SO, stratum oriens; CA1, CA2, and CA3, fields of the hippocampus; DG, dentate gyrus, lateral blade; Hilus, dentate gyrus, hilar blade

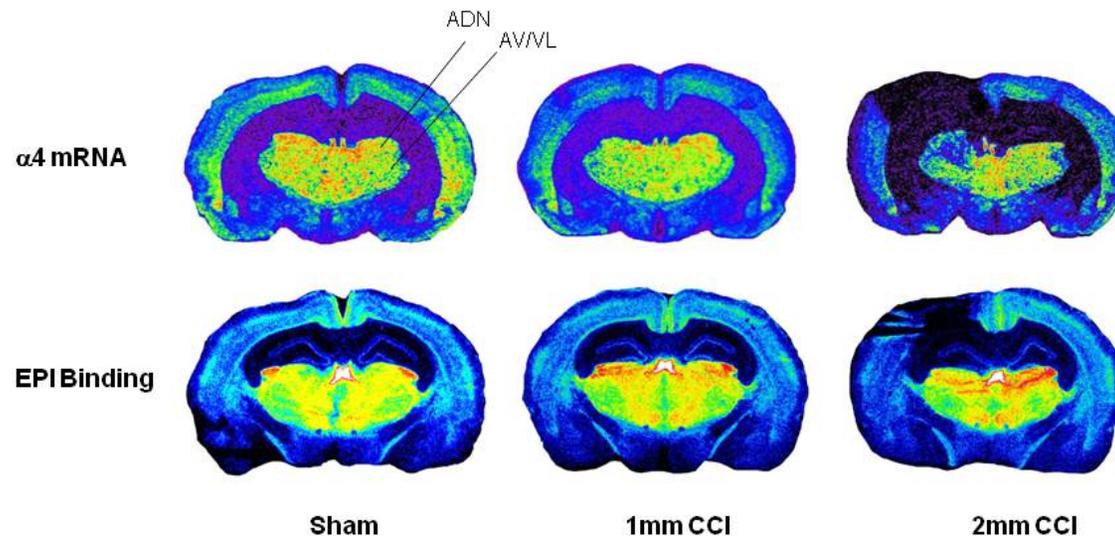
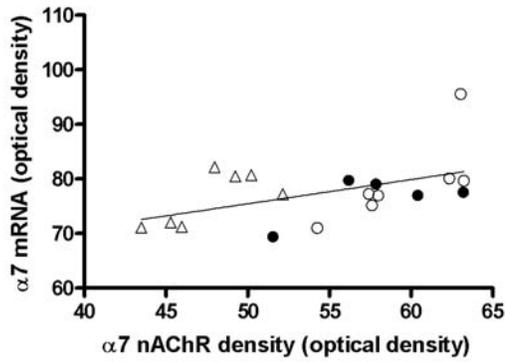
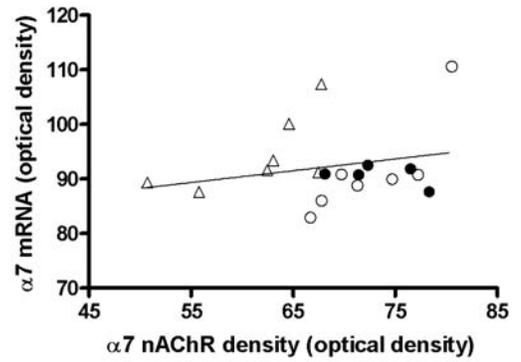


Figure 4.2. $\alpha 4$ nAChR mRNA is reduced in thalamic nuclei following TBI. Representative autoradiographs demonstrate $\alpha 4$ mRNA as measured by *in situ* hybridization and non- $\alpha 7$ nAChR density determined by [125 I]-EPI binding. The left side of the brain shown is the ipsilateral side. Abbreviations: ADN, anterodorsal thalamic nucleus; AV, anteroventral thalamic nucleus; VL, ventrolateral thalamic nucleus. Note that the contralateral side was used as a control for each brain section in order to account for differences in background density visible herein.

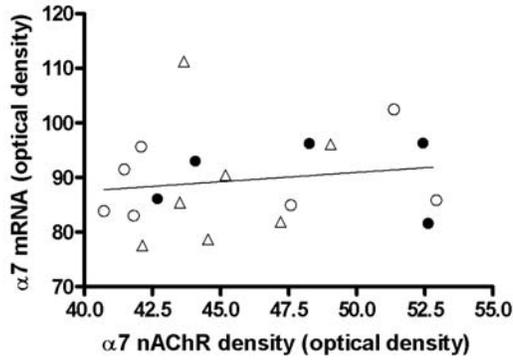
Auditory Cortex Layers 1-4



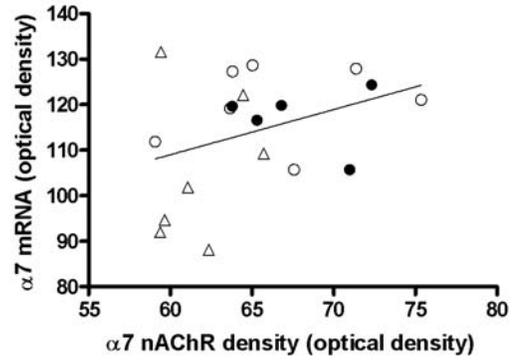
Auditory Cortex Layers 5-6



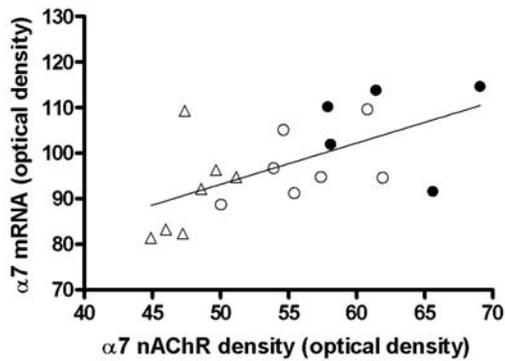
Hippocampus, CA1 Layer



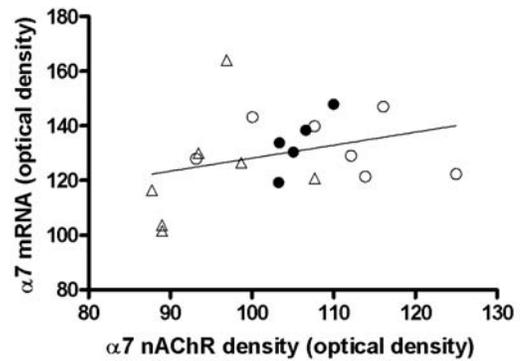
Hippocampus, CA3 Layer



Dentate Gyrus, Lateral Blade



Dentate Gyrus, Hilar Blade



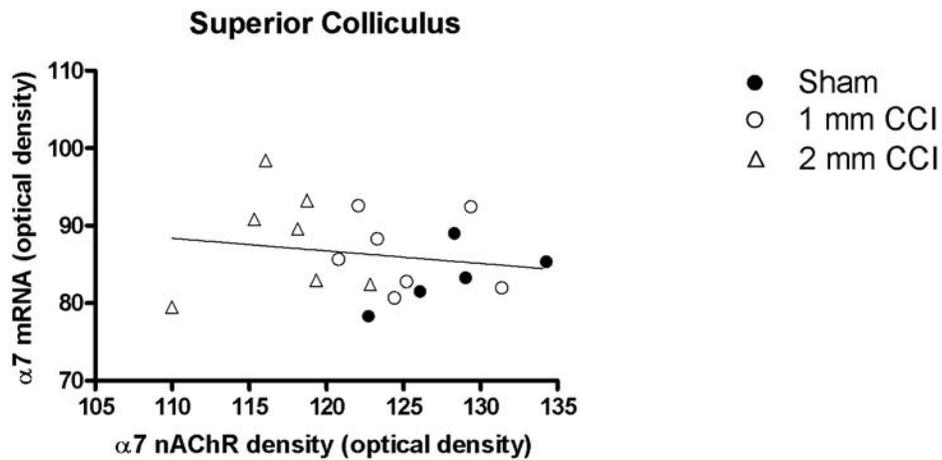


Figure 4.3. Correlation analysis of $\alpha 7$ nAChR density and $\alpha 7$ nAChR mRNA in brain regions demonstrating alterations in rats 48 hours following TBI. Data used for the correlation are measurements taken from the ipsilateral side of the brain from rats in the 2 mm CCI, 1 mm CCI, and sham operated groups. Pearson correlation results are as follows:

Auditory cortex layers 1-4: $r=0.4911$, $p=0.0328^*$

Auditory cortex layers 5-6: $r=0.2384$, $p=0.3257$

Hippocampus, layer CA1: $r=0.1616$, $p=0.5088$

Hippocampus, layer CA3: $r=0.3595$, $p=0.1306$

Dentate gyrus, lateral blade: $r=0.6001$, $p=0.006^*$

Dentate gyrus, hilar blade: $r=0.3166$, $p=0.1867$

Superior colliculus: $r=-0.1780$, $p=0.4661$

*denotes significant Pearson correlation coefficient (r)

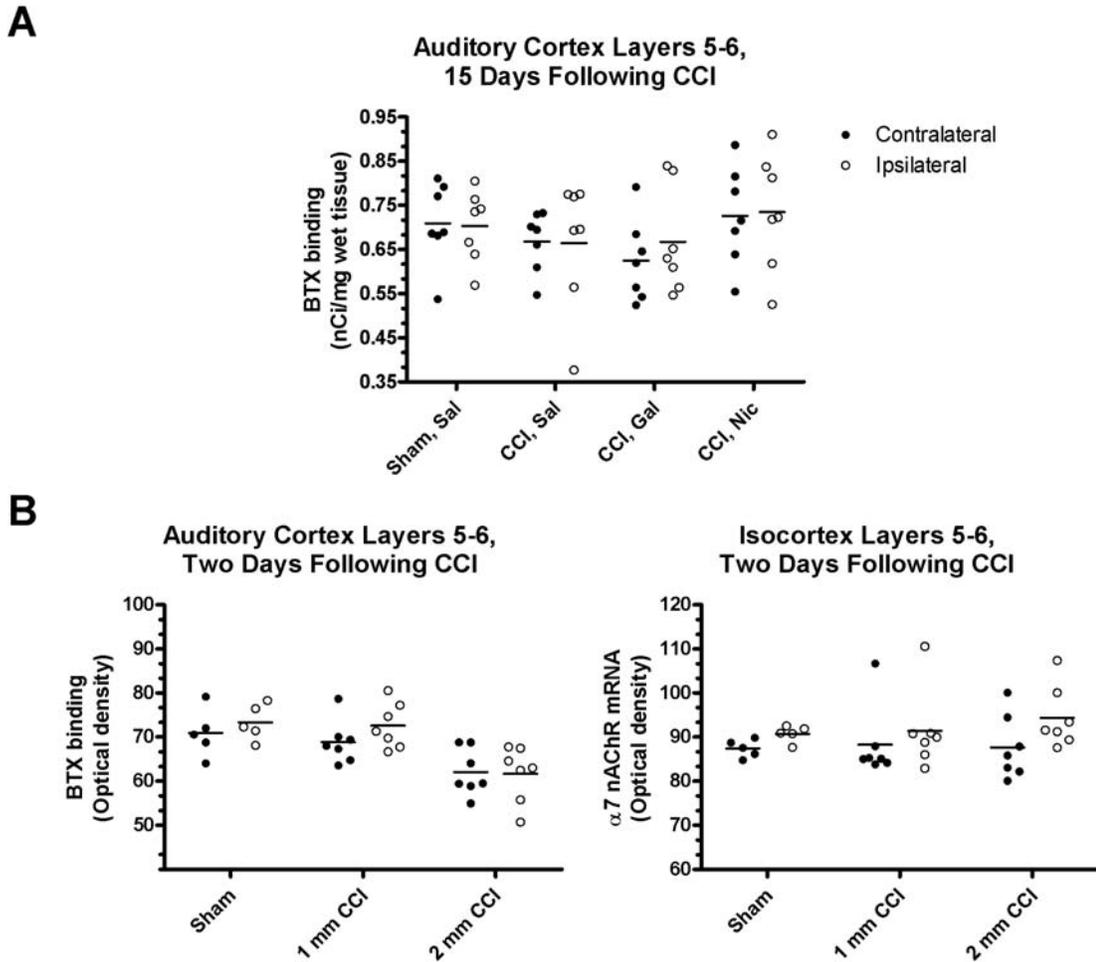


Figure 4.4. Upregulation of $\alpha 7$ mRNA occurs in the auditory cortex, layers 5-6 following TBI. Panel A includes the results of the BTX binding analysis from the study of galantamine following TBI (Chapter 3) for comparison and Panel B shows the results of the BTX binding and $\alpha 7$ *in situ* hybridization studies 48 hours following TBI. The ipsilateral and contralateral measurements for the $\alpha 7$ *in situ* hybridization analysis were not presented in the current chapter but can be found in Appendix I. Two-way repeated measures ANOVA revealed a significant effect of side in the Auditory cortex layers 5-6, $F(1,16) = 25.85$, $p < 0.0001$, for $\alpha 7$ mRNA (Panel B, right).

Chapter 5: Summary and Conclusions

In the dissertation, *Alpha7 Nicotinic Acetylcholine Receptor Regulation in Experimental Neurodegenerative Disease*, experimental models of Alzheimer's disease and traumatic brain injury were employed in order to evaluate alterations in nicotinic receptors in neurodegenerative disease states involving common neurobiological processes. The research herein also investigated the effects of nAChR modulation on changes associated with neurodegeneration and the regulation thereof. Three separate research questions were investigated in the dissertation:

1. Is an increase in the density of $\alpha 7$ nicotinic acetylcholine receptor an early marker in an experimental mouse model of Alzheimer's disease?
2. Does galantamine, a commonly used pharmacotherapy for functional improvement in AD patients, target nicotinic receptor deficits to improve cognitive impairment following experimental brain injury?
3. Are $\alpha 7$ nicotinic acetylcholine receptor protein deficits accompanied by deficits in mRNA expression following experimental traumatic brain injury?

The objective of the first research study, "Upregulation of hippocampal $\alpha 7$ nAChRs in a transgenic mouse model of Alzheimer's disease" was to investigate alterations in cholinergic receptor expression in an Alzheimer's mouse model as a function of age and A β deposition. In this study, nAChR and mAChR expression was evaluated using quantitative receptor autoradiography in a transgenic mouse model that overexpresses APP. This study is the first to use a longitudinal approach to comprehensively assess cholinergic receptor changes and map these changes throughout the brain with the use of autoradiography.

The original objective to correlate A β deposition with changes in nAChR was not possible because unexpectedly, no amyloid deposits were found in mice tested out to 18 months of age. However, we presumed that these mice were exposed to higher than normal soluble A β levels, as previous studies showed a two fold increase in APP expression (Borchelt et al., 1996; Borchelt et al., 1997; Borchelt et al., 2002) and elevated A β levels (Price et al., 1998). Thus, changes in nAChR density in APP^{swe} mice suggest

an amyloid based mechanism and are probably due to elevated soluble A β in this strain compared to wild-type mice.

This study showed a significant increase in BTX binding in various regions in APP^{swe} mice with age compared to wild-type mice. These findings are consistent with our hypothesis and other reports (Dineley et al., 2001; Bednar et al., 2002; Dineley et al., 2002b; Hellstrom-Lindahl et al., 2004b) that demonstrate elevated $\alpha 7$ binding in Alzheimer's mice and may be due to agonist properties of A β acting at the $\alpha 7$ nAChR (Dineley et al., 2002a; Dougherty et al., 2003). When examining genotype only, BTX binding was significantly elevated in hippocampal regions in APP^{swe} mice, including anterior hippocampal layers CA1, CA2, and posterior hippocampal layer CA3. These findings are consistent with previous studies that suggest a sparing or elevation in cholinergic neurotransmission occurring early in the course of disease (Gilmor et al., 1999; DeKosky et al., 2002; Ellis et al., 2008; Ikonovic et al., 2009). This is therapeutically significant because the preservation of receptors early in the disease process may allow for pharmacological interventions targeted at the $\alpha 7$ nAChR. Once receptors are lost, the presumption of neuronal cell death precludes the use of pharmacotherapeutic strategies aimed at enhancing cholinergic neurotransmission. Interestingly, in the Tg2576 mouse model of AD, enhanced nicotinic receptor binding occurred in conjunction with an upregulation in the mRNA levels of both $\alpha 7$ and $\alpha 4$ receptor subunits (Bednar et al., 2002), and in patients with mild to moderate AD, $\alpha 7$ mRNA was upregulated in one report (Counts et al., 2007). This suggests that the agonist properties of A β at nAChRs may involve transcriptional mechanisms. Evaluation of mRNA expression by *in situ* hybridization of $\alpha 7$ and $\alpha 4$ in APP^{swe} and wild-type mice would be beneficial in further characterizing the effects of AD on nAChR changes.

It is possible that the increase in soluble A β as seen in animal models of AD and in early AD causes changes in neurotransmitter receptor expression. In normal brain physiology, A β 42, in picomolar concentrations, is involved in synaptic plasticity and has recently been shown to involve the $\alpha 7$ nAChR (Puzzo et al., 2008). In early Alzheimer's disease, as A β concentrations begin to elevate, nAChRs may upregulate as a short term compensatory mechanism to counter a loss of cholinergic innervation that contributes to

cognitive impairment. The increased levels of soluble A β elicit agonist properties at the α 7 and possibly non α 7 nAChRs which in turn causes upregulation of nAChR. It is possible that upregulation of nAChRs is a mechanism by which enhanced cholinergic neurotransmission may facilitate the regulation of APP processing. Moreover, upregulation may be a means to promote neuroprotection (Jonnala and Buccafusco, 2001). To date, it is not known if upregulation results in functional receptors or elevated levels of inactive receptors, as some studies demonstrate neuroprotection due to the activation of nAChRs (Dajas-Bailador et al., 2000). Other reports have shown that blockade may provide this benefit, namely deletion of the α 7 nAChR gene in aged APP overexpressing mice (PDAPP) resulting in cognitive improvement in the Morris water maze and maintained synaptic integrity (Dziewczapolski et al., 2009). Additionally, A β activation of nAChRs may cause enhanced Ca²⁺ influx and Ca²⁺ dysregulation as well as the accumulation of intracellular A β . Both of these effects further the cholinergic derangement as seen by changes in nAChR and mAChR expression, decreases in cholinergic activity, and the selective loss of cholinergic neurons in AD and AD models. Hence, there is no simple answer as to how A β modulates the nicotinic receptor.

A new hypothesis proposed by Hernandez and colleagues (2010) states soluble, oligomeric A β activation of α 7 nAChRs results in neuroprotection in early AD through preservation of cholinergic neurotransmission and prevention of further toxic effects of A β . This supposition is supported by results from studies employing an α 7 nAChR gene deletion in Tg2576 mice. Tg2576 mice lacking the α 7 gene display greater cognitive impairment and cholinergic dysfunction and elevated soluble A β levels compared to mice with an intact α 7 nAChR at 5 months of age when cognitive decline begins (Hernandez et al., 2010). Activation of α 7 nAChRs leads to Ca²⁺ and ERK MAPK activation promoting learning and memory (Dineley et al., 2001; Hernandez et al., 2010). The authors also propose that with the accumulation of A β and subsequent progression of disease, A β and α 7 nAChRs bind irreversibly resulting in α 7nAChR inactivation, synaptic dysfunction, and a worsening of disease features (Hernandez et al., 2010). By preventing the association of A β with nAChR, the use of a nAChR partial agonist, S 24795, has demonstrated enhanced α 7nAChR function and synaptic plasticity in studies

using postmortem brain tissue from AD patients (Wang et al., 2009). S 24795 also improved $\alpha 7$ nAChR function and reduced tau phosphorylation and A β accumulation in rodent brain tissue exposed to A β (Wang et al., 2010).

Furthermore, a downregulation in BTX binding occurred in APP^{swe} in other brain regions. Thus, the relative changes in binding are region specific. Due to the predominant presynaptic location of $\alpha 7$ nAChRs and widespread distribution throughout the CNS, it is possible that A β may affect this receptor differently depending on brain regions and local concentrations. Minimal changes were seen in EPI binding demonstrated by slight decreases in some regions. The downregulation in EPI binding seen in some regions as well as that seen from BTX binding may be the result of a loss of cholinergic innervation. While other studies have shown decreases in $\alpha 4\beta 2$ nAChRs (Apelt et al., 2002) as well as in $\alpha 4$ mRNA (Mousavi et al., 2004), the current results reinforce the selective vulnerability of $\alpha 7$ nAChRs in AD models demonstrated repeatedly in the literature. The data herein suggest the $\alpha 7$ nAChR is a sensitive target for regulation by A β in mice that overexpress APP.

The ability to draw definitive conclusions from this study would have been strengthened by the measurement of soluble A β in the brains of these mice to better correlate the cholinergic receptor changes with disease progression as well as in the studies in experimental TBI. In addition, assessing cognition in the AD model using behavioral tasks, such as the Morris water maze or passive avoidance, could help characterize this model at various ages when compared to nicotinic receptor alterations. Other studies that would be useful include *in situ* hybridization for nicotinic receptor mRNA and functional receptor studies to assess the activity of altered receptors. The measurement of ChAT activity may help to compare changes in this model to early changes occurring in MCI. Also, evaluation of corticosterone levels could help explain deficits in nAChR expression noted in some regions. Lastly, the widely employed Tg2576 Alzheimer model could be used to further assess nicotinic receptor changes at early ages and nicotinic receptor targeted therapies. Additionally, longitudinal studies using Tg2576 mice lacking the $\alpha 7$ nAChR could be used to determine when the

transition occurs between the neuroprotective upregulation of $\alpha 7$ nAChRs and the loss of function and/or density that is found in late stage AD patients.

The potential clinical relevance of these findings deserves further examination. A complete characterization of the neuropathological processes in AD and how they impact cognitive function, beginning in the earliest stages through end stage disease is warranted. A longitudinal study involving PET analysis of presynaptic cholinergic markers, nAChRs, and A β concentrations investigating healthy individuals, patients with MCI, and patients with early, mild AD would be highly valuable in terms of assessing cause and effect relationships. Patients would be followed long term and changes in cholinergic neurotransmission could be determined as patients age normally, patients with MCI show progression to AD or do not, and as patients with mild AD progress to more severe forms of disease. These measurements would then be compared with the results of psychometric examinations to assess cognition. Similar methods have previously been employed; however, each study has addressed patients at a static time point in the progression of AD (Nordberg et al., 1997; Ellis et al., 2008). These methodologies have also been employed to evaluate the effects of AChEIs in the treatment of AD (Kadir et al., 2008) and could be employed longitudinally to assess a more extensive range of nAChR modulation strategies.

A further investigation of nAChR regulation in neurodegenerative disease was employed using experimental TBI because of similarities in the pathophysiology with Alzheimer's disease and the well documented association between TBI and AD. The purpose of the second research study, "Galantamine treatment following traumatic brain injury in rats: effects on cognition and nAChR expression" was to evaluate the effects of the nAChR positive allosteric modulator, galantamine, on cognition and nicotinic receptor expression in rats following TBI. This study evaluated the effects of galantamine treatment following CCI on the Morris water maze behavioral task and on nicotinic receptor expression assessed by quantitative autoradiography. The first study tested a 15 day regimen of galantamine, while the second study investigated galantamine treatment effects on the Morris water maze swim task at different time points following TBI.

Interestingly, the results of the EPI binding analysis demonstrate mild to moderate experimental TBI impairs the ability of nAChR modulation to induce receptor upregulation. Galantamine treated rats displayed significant reductions in EPI binding and did not show increases in the contralateral brain as expected. Interestingly, while nicotine treated rats showed upregulation of EPI binding contralaterally compared to sham operated rats in some brain regions, indicating the ability to upregulate the uninjured brain, some brain regions demonstrated deficits on the injured side of the brain. These results suggest that TBI may interfere with galantamine and nicotine mediated upregulation.

In one study, administration of galantamine 2 mg/kg twice daily subcutaneously for 10 days caused an upregulation in [³H]-cytisine binding in hippocampus and BTX binding in the cortex of mice (Svedberg et al., 2004), but a PET study of AD patients treated with galantamine showed no changes in [¹¹C]-nicotine binding in the cortex (Kadir et al., 2008). In rodents, chronic nicotine exposure caused an upregulation in nAChRs (Marks et al., 1983; Schwartz and Kellar, 1983), and rats receiving a choline supplemented diet for two weeks demonstrated a selective upregulation in $\alpha 7$ nAChRs in cortical and hippocampal brain regions (Guseva et al., 2006). Additionally, the pattern of upregulation by nicotine has been shown to depend on receptor subtype. For example, $\alpha 6\beta 4$ nAChRs upregulate at different doses and exposure times compared to $\alpha 4\beta 2$ nAChRs (Walsh et al., 2008). Physiologic changes occurring in TBI such as altered cerebral blood flow could affect the concentration, distribution, and pharmacodynamic properties of nAChR agonists within various brain regions. TBI may interfere with any one of the processes involved in nAChR upregulation including transcription, receptor subunit assembly, post-translational modifications or desensitization; therefore, evaluation of mRNA expression would help to characterize alterations in upregulation following TBI.

Previous studies from our laboratory showed that a choline supplemented diet attenuated $\alpha 7$ nAChR deficits following CCI and provided significant cortical tissue sparing but not a substantial improvement in Morris water maze performance (Guseva et al., 2008). Galantamine's potential cognitive enhancing properties were evaluated in experimental brain injury. However, galantamine did not provide a significant

improvement in the Morris water maze or the cortical sparing analysis with the dosing regimen tested. Further studies with larger sample sizes may be needed to determine if facilitation of neurological recovery following experimental TBI can be demonstrated by galantamine.

The possible utility of galantamine in the clinical setting is dependent on a risk/benefit assessment of drug administration in patients with mild to moderate TBI. Adverse reactions of galantamine include nausea, vomiting, dizziness, depression, anorexia, and weight loss (Defilippi and Crismon, 2003; Robinson and Plosker, 2006; Ortho-McNeil Neurologics, 2008). Additionally, due to its cholinomimetic properties, galantamine may cause bradycardia, atrioventricular block, and seizures, which is of concern due to the elevated risk of seizure disorder following moderate and severe TBI. As galantamine is partially metabolized by CYP2D6 and CYP3A4, drug interactions may occur with other medications employed in this setting. For example, TBI induced depression treated with paroxetine may raise galantamine concentrations exacerbating adverse effects by inhibiting CYP metabolism (Defilippi and Crismon, 2003; Blennow et al., 2006). Additionally, it is possible that elevated galantamine concentrations may also occur due to depressed cytochrome P450 activity following TBI, depending on severity, as decreases in drug metabolism have been demonstrated in human subjects following an acute phase response (Shedlofsky et al., 1994). By contrast, one study showed enhanced clearance of cyclosporine A, primarily metabolized by CYP3A4, in patients with severe TBI (Empey et al., 2006). It is not known how these changes would affect galantamine treatment of milder forms of TBI. Of note, in 2005 the FDA issued an alert regarding two clinical trials in MCI patients assessing galantamine's ability to slow the progression to AD. These trials resulted in a greater number of deaths in the MCI group compared to the placebo group. After a review process, the FDA did not find enough evidence to alter galantamine prescribing, but did note that galantamine is only approved for use in patients with mild to moderate AD (FDA, 2005). The benefits of galantamine have been reviewed in detail herein. Galantamine's preclinical neuroprotective and anti-inflammatory properties and clinical efficacy in the treatment of AD symptomatology support a potential clinical benefit in TBI. Additional studies are needed to determine if the benefits outweigh the risks in the evaluation of galantamine for treatment in TBI.

Likewise, continuing research of other nicotinic receptor modifying drugs as possible pharmacotherapies for cognitive impairment following TBI is warranted.

Furthermore, the addition of galantamine brain concentrations and the determination of acetylcholinesterase inhibition may improve future dosing regimens and pharmacodynamic correlations. Possible dose and timing issues require further exploration, as the dose used herein was chosen based on the literature in which differences in rat strains and experimental conditions may hinder a correct selection. Additionally, performing the Morris water maze or other behavioral task on naïve rats treated with galantamine or the inclusion of a sham operated, galantamine treated group would allow for a baseline cognitive effect by which to assess treatment. Furthermore, a galantamine pretreatment regimen causing upregulation of receptors prior to a CCI injury may help elucidate the effect that TBI has on nAChR upregulation. The mechanism by which TBI interferes with upregulation is unknown but may involve the regulation of mRNA expression, and thus, investigations into the transcriptional regulation of nAChRs in TBI are needed. It is possible that a reduction in mRNA in TBI leads to fewer receptors available to desensitize and undergo upregulation. Because of galantamine's therapeutic benefit in patients with AD, continuing research is necessary to determine if this drug or other modulators of nAChRs can benefit those patients with lasting cognitive impairments due to traumatic brain injury.

The aim of the third research study, "Experimental traumatic brain injury reduces the expression of hippocampal $\alpha 7$ nicotinic receptor mRNA," was to determine if decrements in $\alpha 7$ nAChR expression correlate with changes in mRNA expression following TBI. Additionally, we were interested in obtaining further mechanistic information needed to explain the impaired plasticity of non- $\alpha 7$ nAChRs demonstrated in the previous examination of galantamine following experimental TBI. In this study, *in situ* hybridization and receptor autoradiography with semi-quantitative analysis were performed to evaluate $\alpha 7$ and $\alpha 4$ nAChR mRNA expression two days following experimental TBI, a time when previous studies observed consistent decreases in nAChR expression.

Previously our laboratory demonstrated significant reductions in hippocampal and cortical $\alpha 7$ nicotinic receptor binding in rats subjected to CCI (Verbois et al., 2000). The

results of this study are the first to show downregulation of $\alpha 7$ mRNA accompanying reductions in $\alpha 7$ nAChR binding in rats 48 hours following a moderate CCI. Deficits in $\alpha 7$ mRNA occurred along with reductions in BTX binding in multiple brain regions including several hippocampal subfields, the dentate gyrus, and superior colliculus. In addition, $\alpha 4$ mRNA was reduced in the thalamus, and deficits occurred along with diminished EPI binding in the metathalamus. The $\beta 2$ subunit was not assayed in this study, and including it in the analysis would be useful in the future to further characterize the effect of TBI on nAChRs as most non- $\alpha 7$ nAChR in the mammalian brain are of the $\alpha 4\beta 2$ subtype. It is possible that loss of receptors and mRNA are due to the excitotoxic processes involved in TBI including abnormal Ca^{2+} signaling, mitochondrial dysfunction, free radical damage, and resulting neurotoxicity. But evidence from the numerous studies of nAChR changes in neurodegenerative disease suggests a selective targeting of this receptor type. Further studies of the mRNA expression in other types of neurotransmitter systems would be useful.

TBI results in a secondary delayed injury involving ischemia, Ca^{2+} mediated excitotoxicity, mitochondrial dysfunction, free radical production, cell death, and inflammation (Bramlett and Dietrich, 2007; Greve and Zink, 2009). Additionally, several studies have demonstrated elevations in $\text{A}\beta$ following TBI (Gentleman et al., 1993; Olsson et al., 2004) and repetitive mild brain trauma hastens $\text{A}\beta$ accumulation and increases $\text{A}\beta 42$ and $\text{A}\beta 40$ concentrations in nine month old Tg2576 mice compared to a single injury (Uryu et al., 2002). The presence of elevated levels of $\text{A}\beta$ following TBI may result in upregulated $\alpha 7$ mRNA and $\alpha 7$ nAChR in localized brain regions, as $\alpha 7$ mRNA was elevated in the inner layers of the cortex in the current study. There are reports of upregulated mRNA in the hippocampus (Hellstrom-Lindahl et al., 1999) and nucleus basalis (Counts et al., 2007) in AD. In theory, it is also possible that the presence of elevated $\text{A}\beta$ following TBI could compete with nAChRs administered exogenously. In the evaluation of galantamine and nicotine following TBI, the impairment of upregulation could in part be due to the presence of relatively low concentrations of $\text{A}\beta$ acting at nAChRs preventing the expected upregulation of nAChRs following agonist administration. A time course of mRNA expression, $\text{A}\beta$ concentrations, and nicotinic

receptor functional studies following TBI may provide additional insight into the mechanisms involved in TBI related nAChRs changes.

Our laboratory previously found no significant effect of the $\alpha 7$ nAChR gene deletion on cortical tissue sparing or a marker of brain inflammation following TBI in studies using $\alpha 7$ nAChR knock out mice (Kelso et al., 2006). Given the recent finding in Tg2576 mice lacking the $\alpha 7$ nAChR gene purporting soluble, oligomeric A β activation of $\alpha 7$ nAChRs resulting in neuroprotection in early AD (Hernandez et al., 2010), a further study is proposed involving experimental TBI in this model. In this study, CCI performed at early and late time points in AD related disease pathology could help elucidate the expression and function of nAChRs as well as their effect on functional improvement. In this design, elevated soluble A β may regulate nAChR plasticity and effect on functional outcome differently depending on age.

In conclusion, two models of neurodegenerative disease, experimental Alzheimer's disease and traumatic brain injury, display regional dependent changes in $\alpha 7$ nAChRs. AD and TBI may involve complex but parallel processes contributing to the regulation of $\alpha 7$ nAChRs. Further studies are needed to better comprehend the intricate mechanisms involved in neurodegenerative disease and to guide the development of pharmacotherapies in the clinical setting. The research in this dissertation may contribute to the overall understanding of nAChR regulation in diseases involving neurodegenerative processes.

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Appendix

Alterations in $\alpha 7$ nAChR mRNA following TBI in rats measured by *in situ* hybridization. Data are expressed as group mean \pm standard deviation optical density.

Brain Region	Sham		1 mm CCI		2 mm CCI	
	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral
Neocortex						
Auditory cortex layers 1-4	76.80 \pm 1.96	76.57 \pm 4.14	78.79 \pm 7.84	79.41 \pm 7.75	78.10 \pm 7.23	76.42 \pm 4.89
Auditory cortex layers 5-6	87.41 \pm 2.01	90.75 \pm 1.88	88.27 \pm 8.22	91.42 \pm 8.93	87.65 \pm 7.22	94.38 \pm 6.96*
Basal ganglia						
Endopiriform	97.34 \pm 3.39	101.44 \pm 4.08	97.25 \pm 7.58	97.94 \pm 11.00	104.25 \pm 11.67	107.83 \pm 14.55
Anterior hippocampus						
CA1 layer	91.77 \pm 4.47	90.65 \pm 6.55	93.53 \pm 10.18	89.61 \pm 7.29	94.65 \pm 9.05	88.75 \pm 11.89
CA2 layer	67.82 \pm 1.59	69.46 \pm 0.85	69.63 \pm 5.48	70.23 \pm 4.81	70.89 \pm 5.86	68.23 \pm 5.45
CA3 layer	116.25 \pm 8.96	117.26 \pm 7.01	121.76 \pm 9.74	120.27 \pm 8.79	124.38 \pm 12.63	105.66 \pm 16.28*
Dentate gyrus, lateral blade	108.51 \pm 9.07	106.46 \pm 9.71	106.91 \pm 8.18	97.26 \pm 7.50*	105.32 \pm 9.24	91.35 \pm 10.03*
Dentate gyrus, hilar blade	138.46 \pm 10.94	133.95 \pm 10.50	140.51 \pm 11.24	133.00 \pm 10.28*	145.81 \pm 16.99	123.24 \pm 20.92*
Posterior hippocampus						
Dentate gyrus, hilar blade	157.34 \pm 2.04	157.37 \pm 2.81	157.80 \pm 6.48	157.82 \pm 6.69	159.33 \pm 12.84	155.35 \pm 10.52
Mesencephalon						
Superior colliculus	83.02 \pm 2.59	83.50 \pm 4.03	87.61 \pm 4.77	86.36 \pm 4.89	91.25 \pm 6.99	88.14 \pm 6.78*

* denotes significantly different from the contralateral side

Statistical analysis was performed using a two-way (injury, side), repeated measures, ANOVA followed by a Tukey-Kramer multiple comparisons test (GBSTAT software) ; significance was set at $\alpha = 0.05$.

Auditory cortex layers 5-6, $F(1,16) = 25.85$, $p < 0.0001$

CA3 layer, $F(1,16) = 11.69$, $p = 0.0035$

Dentate gyrus, lateral blade, $F(1,16) = 73.66$, $p < 0.0001$

Dentate gyrus, hilar blade, $F(1,16) = 93.45$, $p < 0.0001$

Superior colliculus, $F(1,16) = 13.63$, $p < 0.002$

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Charriez CM, Scheff SW, Pauly JR. The effects of galantamine on cognition following traumatic brain injury in rats. Abstract presented at the National Neurotrauma Symposium, November 2005.

Charriez CM, Marks MJ, Scheff SW, Pauly JR. Experimental rat brain injury reduces the expression of hippocampal $\alpha 7$ nicotinic receptor mRNA. Abstract presented at the Society for Neuroscience Annual meeting, October 2004.

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