GABAERGIC SYSTEMS IN A MODEL OF AGE-RELATED COGNITIVE IMPAIRMENT

A Dissertation

by

CANDI LYNN LASARGE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2011

Major Subject: Psychology

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Approved by:

Chair of Committee,	Jennifer Bizon
Committee Members,	Barry Setlow
	William Griffith
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ABSTRACT

GABAergic Systems in a Model of Age-Related Cognitive Impairment.

(May 2011)

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Chair of Advisory Committee: Dr. Jennifer L Bizon

With medical advancements extending the life span, age-related cognitive decline is a growing problem for the United States. A rat model of cognitive aging was used to investigate the GABAergic neurotransmitter system in relation to changes in learning and memory functions. Confocal stereology was used to determine the number of GABAergic and cholinergic projection neurons in the rostral basal forebrain of spatially characterized young and aged male F344 rats. The GABAergic system was then assessed as a potential target for improving age-related cognitive decline using an odor discrimination task sensitive to decline in aging.

Performance of aged rats was impaired compared to young rats on the spatial version of the Morris water maze. Notably, a high degree of variability in individual abilities was observed among aged rats such that some aged rats performed on par with young (aged-unimpaired) and others performed outside the range of young, demonstrating impairment (aged-impaired). The number of

basal forebrain neurons expressing multiple immunomarkers for GABAergic septohippocampal projection cells was selectively increased in aged-impaired rats in comparison to both young and aged-unimpaired rats. Indeed, among aged rats, worse performance in the water maze was reliably associated with higher GABAergic cell number. The number of cholinergic neurons, quantified in adjacent sections did not differ as a function of chronological age or cognitive status. These data suggest that aging can dysregulate GABAergic systems in circuitry important for learning and memory and such alterations may contribute to age-related cognitive decline.

To test whether the GABAergic system may be a viable target for treating age-related cognitive decline, a second cohort of young and aged rats was characterized in an odor discrimination task. Similar to aged rat water maze performance, some aged rats performed odor learning discrimination problems on par with the young cohort (i.e. aged-unimpaired) and some aged rats were impaired compared to young (i.e. aged-impaired). Using a within-subjects design, the GABA(B) antagonist, CGP 55845 completely ameliorated odor discrimination learning deficits in aged-impaired rats in a dose-dependent manner. These data support the hypothesis that the GABAergic system should be a novel target for therapies aimed at treating age-related cognitive decline.

DEDICATION

Chloe, know you can do anything with hard work and determination. Mom, thank you for supporting me in all my endeavors, no matter how painful they were to watch (at least the running events were better than my basketball career). I am grateful for all the people that came into my life during graduate school, and some that left during that time, that allowed me to grow into the person I am today.

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To my committee, Barry Setlow, William Griffith, James Grau, and Mark Packard, thank you for your guidance and support with my dissertation and defense. A special thanks to Barry, who was essential to my training in all behavioral experiments, and to Bill, who provided me with tools necessary for my experiments. I am grateful that such great researchers were a part of my training and career development.

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effort and help from my undergrads that were integral to the completion of these projects: John Hartman, Brian Ambrose, Alyssa Faught, Jennifer Graham, Jeff Mayse, Bryce Gagliano, and many, many more.

Finally, thanks to my family and friends that waited patiently, supporting and motivating me to finish. Jason, thank you for putting up with me while writing this document and helping me to prepare for the defense. I know I put you through a lot of stress, and I am fortunate to have you with me for this completion of grad school and move out of the bubble. Chloe, thank you for all your help through the long years. I know sometimes it was not fun to hang out at home or the lab while mommy worked, but I hope you will remember the fun times with all of the lab family, the games like find the neuron, and your “hands-on” learning about the brain.

NOMENCLATURE

ACh	acetylcholine
AChE	acetyl cholinesterase
AD	Alzheimer's disease
BF	basal forebrain
ChAT	choline acetyl transferase
DB	Diagonal Band of Broca
EC	entorhinal cortex
F344	Fischer 344
GABA	γ-Aminobutyric acid
GAD	glutamic acid decarboxylase
hDB	horizontal limb of the Diagonal Band of Broca
IPSP	inhibitory post-synaptic potential
MCI	Mild Cognitive Impairment
MOB	main olfactory bulb
MRI	magnetic resonance imaging
MS	medial septum
MTL	medial temporal lobe
NDS	normal donkey serum
PARV	Parvalbumin
PET	Positron Emission Tomography

SLI	Spatial Learning Index
TBS	tris-buffered saline
vDB	vertical limb of the Diagonal Band of Broca

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CHAPTER I

INTRODUCTION: GABAERGIC SYSTEMS IN COGNITION AND AGING

Advancements in medical sciences and technology have contributed to a significant increase in the human lifespan over the last half century, from approximately 68.2 years in 1950 to 77.9 years in 2007 (Xu et al., 2010). With more people living to advanced ages in the United States, the elderly population is expected to escalate from 35 million in 2000 to over 70 million by the year 2030 (Federal Interagency Forum on Aging-Related Statistics, 2004). The ability to maintain cognitive function into advanced age is a major concern from both an individual and a public health perspective. Severe dementia resulting from pathological conditions such as Alzheimer's disease (AD) will impact 7 – 8% of aged individuals and is the 6th leading cause of death in Americans; however, a much larger number of the elderly will experience cognitive decline that substantially impacts quality of life without the manifestation of age-related disease (Freedman et al., 2002; Xu et al., 2010). In order to prevent or pharmacologically target age-related cognitive decline, it is critical to thoroughly understand the neurobiological underpinnings of deficits in learning and memory at advanced ages.

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This dissertation follows the style of *Neuroscience*.

Naturalistic variability in aging

Although many people will experience a decline in cognitive function with advancing age, this decline is not an inevitable consequence of the aging process. The natural aging progression in humans shows substantial variability in the decline of mnemonic abilities such that some people begin to show impairments in their fourth decade while others perform on par with young adults past their seventieth year of life (Albert, 1993; Small, 2001; Bizon and Nicolle, 2006). Naturalistic rat models demonstrate a similar variability in cognitive performance at advanced ages, particularly in the domain of spatial reference memory. Some aged rats perform as well as young cohorts in tasks like the water maze, whereas others perform outside the range of young, demonstrating impairment (Gallagher et al., 1993; for review see LaSarge and Nicolle, 2009). Thus, not all aged rats show a decline in spatial learning performance, allowing for their reliable separation into two groups that from here on will be defined as “aged-unimpaired” and “aged-impaired” (LaSarge et al., 2007; Bizon et al., 2009). Understanding the reasons that some aged rats maintain mnemonic function well into advanced ages while others are impaired can aid in understanding human age-related cognitive decline and help to determine new pharmacological therapies to halt or reverse cognitive deficits.

Age-related impairment in memory and olfaction

Impairments associated with explicit memory (i.e. the ability to encode and recall information about people, places, and things) and olfactory function are among the earliest and most widely reported deficits associated with the aging process (Gabrieli, 1996; Freedman et al., 2002). A large amount of literature suggests that basal forebrain (BF) neurons that project to both MTL and olfactory bulb targets are vulnerable to changes with age, including death, and that these projections are important for cognition (Fischer et al., 1991; Mufson et al., 2002; Smith et al., 2004). It is now well-established that explicit/ spatial memory deficits can occur even in the absence of frank neural loss in medial temporal lobe structures (Rapp and Gallagher, 1996; Rapp et al., 2002; West et al., 2004; Shamy et al., 2006). Impairments in declarative memory tasks related to the medial temporal lobe (MTL), such as remembering spatial relationships and landmarks, are common in aged humans. Studies using Positron Emission Tomography (PET) and magnetic resonance imaging (MRI) show that the MTL is one of the brain areas most susceptible to age-related decline in function (Lipman, 1991; Jack et al., 1997; Small, 2001; Rosenzweig and Barnes, 2003; Raz, 2004) . Additionally, olfactory deficits have become recognized as an early indicator of cognitive decline in humans, and a decline in olfactory function is correlated with impairment in 19 cognitive batteries including explicit memory, working memory, and visuospatial ability (Eibenstein et al., 2005; Wilson, 2006).

Septohippocampal anatomy

The medial temporal lobe contains the hippocampal region, consisting of the hippocampal formation and parahippocampal region (for review of hippocampal anatomy see Witter and Amaral, 2004). The hippocampus forms a "C" shaped structure that extends from the septal nuclei of the BF rostrodorsally, over and behind the diencephalon, and caudoventrally in the temporal lobe. The parahippocampal region includes the entorhinal, perirhinal, and postrhinal cortices, located caudal and ventral to the other areas of the hippocampal formation. The entorhinal cortex (EC) that projects to all hippocampal subfields also has bidirectional communication with both perirhinal and postrhinal cortices which, in turn, have afferent and efferent projections to the neocortex, linking the hippocampus to broader circuitry in the brain (see Bizon and Nicolle, 2006).

Projections from the cholinergic and γ-Aminobutyric acid containing (GABAergic) neurons in medial septum (MS) and Diagonal Band of Broca (DB) provide the major innervations from BF to the hippocampal region. MS/DB neurons project to hippocampus and some parahippocampal regions through multiple pathways that include the fimbria and dorsal fornix, which also contain reciprocal projections to the BF (Zaborszky et al., 1999; Butcher and Woolf, 2004; Witter and Amaral, 2004). The fornix splits at the anterior commisure and the rostral extension innervates septal nuclei and other BF structures.

The septohippocampal pathway is comprised of both cholinergic and GABAergic projection fibers that synapse on different target cells. Estimates

indicate that 30-50% of neurons from MS nuclei and 50-75% neurons from DB nuclei that innervate the hippocampus are cholinergic, with GABAergic neurons comprising a large portion of the remaining projection (Witter and Amaral, 2004). There are both excitatory (pyramidal cells) and inhibitory (GABAergic interneurons) neural targets for cholinergic neurons. Cholinergic projections commonly synapse on pyramidal neurons; however, cholinergic synapses have also been identified on other hippocampal neurons including parvalbumin (PARV) containing GABAergic basket cells (Semba, 2000). The role of the cholinergic neurons in cognition has been the focus of a great deal of research given that these cells are profoundly reduced in AD (Johnston et al., 1979; Whitehouse et al., 1982; Coyle et al., 1983; Mann et al., 1984; McGeer et al., 1984; Lowes-Hummel et al., 1989). In contrast, GABAergic BF neurons have been less well characterized within the framework of learning and memory. The focus of this dissertation will be on the septohippocampal GABAergic system in relation to cognitive changes in aging.

GABA in septohippocampal system and cognition

It is postulated that the GABAergic and cholinergic systems of the BF work together to facilitate hippocampal function. Specifically, the balance between these systems has been investigated in association with hippocampal learning and memory. Microdialysis experiments showed hippocampal extracellular acetylcholine (ACh) increases during hippocampal dependent

learning and memory tasks (for example Fadda et al., 1996; Yamamuro et al., 1996; Fadda et al., 2000; McIntyre et al., 2002). Drug treatments that enhance memory have been shown to increase ACh in the hippocampus, and those that impair memory decrease ACh in the hippocampus (Parent and Baxter, 2004). Additionally, GABA released in the BF inhibits the synthesis and release of ACh from BF cholinergic afferents in hippocampus. For example, blockade of GABA(A) receptors in BF via microinfusions of bicuculine results in increased hippocampal choline acetyl transferase (ChAT) activity, the enzyme that joins Acetyl-CoA to choline to make ACh (Kenigsberg et al., 1998). Intraseptal infusions of the GABA(A) agonist muscimol impair measures of memory, including spatial learning, while decreasing hippocampal ACh activity (Brioni et al., 1990; Durkin, 1992). These data suggest that one way which the BF GABAergic system may influence learning and memory is through modulation of hippocampal ACh levels that are correlated with learning and memory.

In addition, some codistributed BF GABAergic neurons directly project to the hippocampus, mainly targeting hippocampal GABAergic interneurons, as shown in Figure 1. These hippocampal GABAergic interneurons in turn project to pyramidal neurons in the hippocampus. Thus, the inhibitory GABAergic projection from BF to hippocampus has the collective result of disinhibiting pyramidal neurons. Age-related changes in the GABAergic BF system, either alone or in concert with alterations in cholinergic neurons, might affect cognitive functions supported by the hippocampus/ MTL system. *Chapter II will investigate*

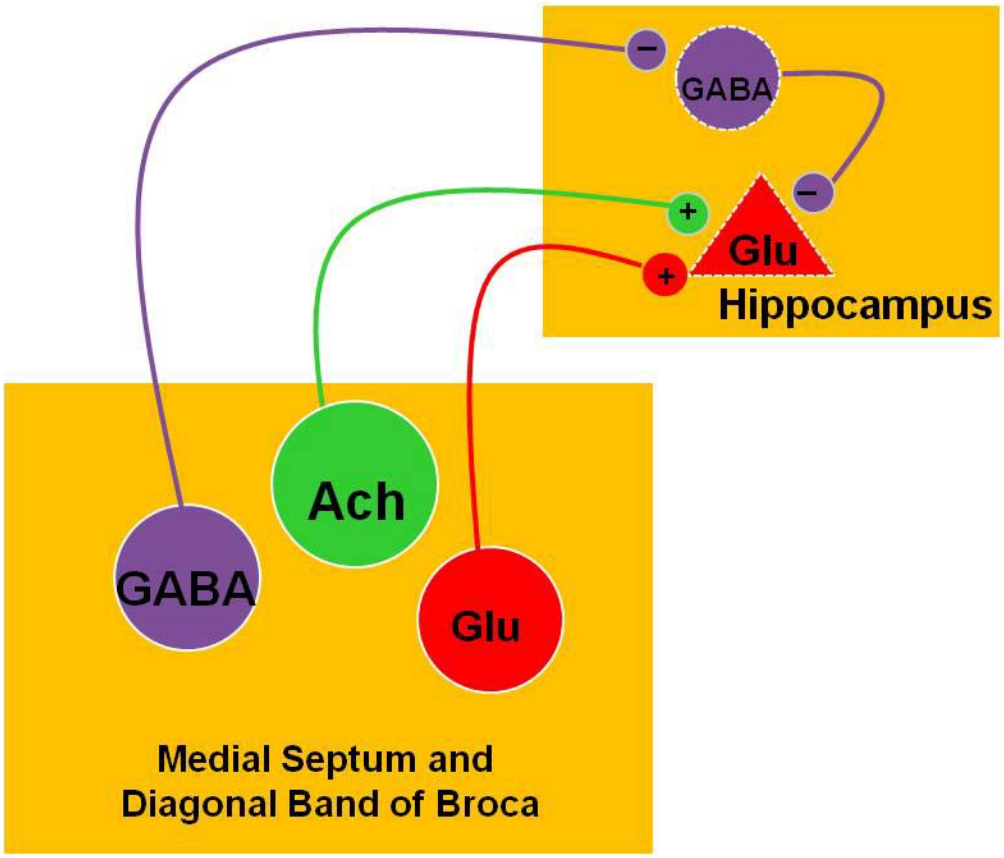

Figure 1. Cholinergic and GABAergic septohippocampal system and projection targets. Cholinergic projection neurons in medial septum and the diagonal band of Broca (MS/DB) synapse on glutamatergic pyramidal cells in the hippocampus. GABAergic projection cells in MS/DB project to GABAergic interneurons in the hippocampus, which in turn synapse onto the pyramidal cells. Inhibitory signals from MS/DB GABAergic cells results in a net disinhibition of pyramidal cells. Additionally, newly discovered glutamatergic projection neurons in the MS/DB have been shown to also synapse on pyramidal hippocampal cells.

age-related changes of GABAergic and cholinergic neurons that comprise the septohippocampal projection.

Olfactory function, memory, and age

Converging evidence suggests that changes in the transduction of odor stimuli and/ or the integration of the olfactory information in the piriform or entorhinal cortex are affected with age. Indeed, recently a simultaneous two-choice odor discrimination task was used to test the relationship between odor discrimination and spatial learning abilities. In LaSarge et al. (2007), Fischer 344 (F344) rats characterized on the spatial water maze were segregated into aged-impaired and aged-unimpaired groups based on their water maze performance in relation to young rats. Upon completion of the water maze, rats were tested in a simultaneous two-choice odor discrimination task. As shown in Figure 2, rats that performed poorly in the water maze (impaired) also demonstrated significantly worse performance on odor discrimination problems when compared to young and aged-unimpaired rats. In contrast, aged-unimpaired rats also performed on par with young in the olfactory discrimination task (LaSarge et al., 2007).

Olfactory system and basal forebrain anatomy

The BF, particularly the horizontal limb of the DB (hDB), provides GABAergic and cholinergic projections to the main olfactory bulb and

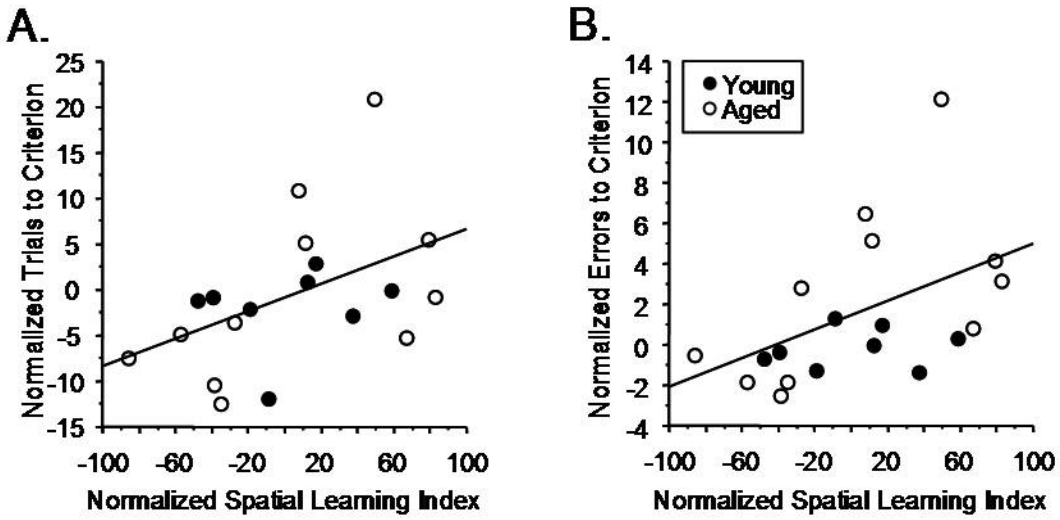

Figure 2*. Scatterplots of individual rat performance, normalized by the mean of each group (young and aged). (A.) Spatial learning index on the water maze vs. mean trials-to-criterion across all three odor discrimination problems. $r=0.47$, $p<0.05$ (B.) Spatial learning index on the water maze vs. mean errors-to-criterion across all three odor discrimination problems. $r=0.48$, $p<0.05$.

consequently is involved in the modulation of the olfactory system (Mesulam et al., 1983; Zaborszky et al., 1999).The olfactory system refers to interconnected structures that are involved in the transduction and processing of odors. Included in this system are the main olfactory bulb (MOB), accessory olfactory bulb, primary olfactory cortex, and accessory olfactory cortex (for review see Shipley et al., 2004). The primary olfactory cortex (including piriform cortex, entorhinal cortex, as well as many other structures) is involved in the processing and integration of odor information (Shipley et al., 2004). In addition to the BF projections to the MOB, there is a high level of interconnectivity between the olfactory system and the MTL. Neurons in the MOB and piriform (olfactory) cortex project directly to EC, which has reciprocal connections with hippocampus (Zald and Pardo, 2000; McNamara et al., 2004; Shipley et al., 2004). Together this circuitry suggests a basis for a strong relationship between olfaction and memory. Furthermore, changes in the integrity of the cholinergic or GABAergic BF systems may have consequences that extend to both the MTL and olfactory system.

The main cholinergic projection to the olfactory system arises from cholinergic nuclei in the hDB of the BF (Shipley et al., 2004). It is estimated that 10-20% of bulbopetal BF projections from hDB are cholinergic (Semba, 2000). Immunohistochemical labeling of acetylcholinesterase (AChE), the enzyme that degrades acetylcholine to choline and acetate, indicates that cholinergic neurons synapse in all layers of the olfactory bulb, as well as piriform cortex (Wilson et

al., 2004). However, ChAT immunohistochemistry revealed no ChAT+ cells in MOB (Shipley et al., 2004; Wilson et al., 2004). This expression pattern suggests that these AChE+ neurons receive cholinergic input and help break down ACh in the MOB, but that ACh is not synthesized in the MOB itself. The utilization of ACh from a source outside the MOB suggests a role of the BF in odor processing.

In addition to the cholinergic afferents from hDB to MOB, 30% of this projection arises from GABAergic nuclei. GABAergic BF neurons that project to MOB are located lateral and caudal to the cholinergic neurons in the hDB (Záborszky et al., 1986; Zaborszky et al., 1999; Semba, 2000). The exact layers of MOB to which GABAergic neurons in the hDB project have not yet been determined. Within the bulb itself there is a large number of GABAergic neurons including interneurons (Shipley et al., 2004). This common location of BF GABAergic terminals and GABAergic interneurons of MOB may also allow for an interaction between the two neuronal populations similar to that in the hippocampus, in which the GABAergic BF neurons might synapse onto GABAergic interneurons. The inhibition of GABAergic cells would result in a net disinhibitory effect on mitral cells, the primary output cells in the MOB.

Cholinergic and GABAergic systems and olfactory learning and memory

Similar to the septohippocampal system, BF cholinergic and GABAergic neurons that project to the olfactory system have been implicated in memory

formation for odorants (Sanchez-Andrade et al., 2005). Although the exact mechanism remains unknown, it is possible that GABAergic BF projections help to disinhibit mitral cells through interaction with presynaptic GABAergic interneurons, while cholinergic projections from BF that synapse on the mitral cells facilitate odor memories. This interaction of cholinergic and GABAergic BF neurons and their projections to MOB are shown in Figure 3. This theory is supported by experiments demonstrating the importance of ACh in MOB for olfactory learning and memory. Lesions of hDB, which provides the main source of ACh, impair olfactory memory (Wilson et al., 2004). Pharmacology studies show that scopolamine, which blocks muscarinic ACh receptors, disrupts memory in olfactory tasks including an olfactory delayed match to sample task (Fletcher and Wilson, 2002). Additionally, ACh infusion into olfactory bulb causes immediate, persistent modulation of olfactory bulb excitability (Wilson et al., 2004). Even less is known about the role of GABA in olfactory discrimination abilities, although GABA(A) and GABA(B) receptors are found throughout most layers of the main olfactory bulb (Shipley et al., 2004). Experiments targeting the GABAergic system in olfactory learning tasks could be of great utility to determine the role of GABA in odor memory and discrimination abilities. *The ability of GABA to modulate odor learning will be examined in Chapter III.*

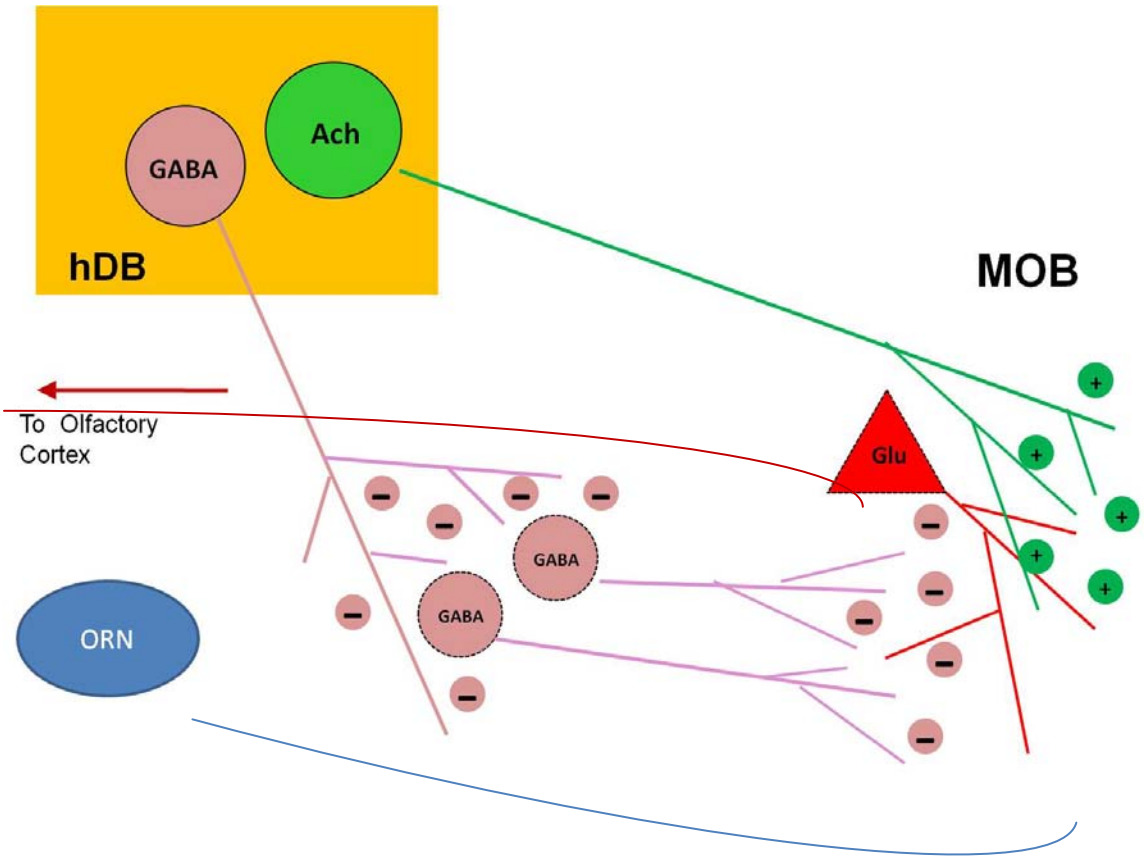

Figure 3. GABAergic and cholinergic (ACh) projections from horizontal limb of the diagonal band of Broca (hDB) to the main olfactory bulb (MOB).
Cholinergic hDB neurons project to glutamatergic mitral cells (Glu) in the olfactory bulb. GABAergic hDB projection neurons synapse on GABAergic interneurons (granule cells), that in turn synapse onto mitral cells. GABA release from hDB GABAergic neurons inhibit granule cells, resulting in a net disinhibition of the mitral cells. Mitral cells receive projections from the olfactory receptor neurons, and are the primary output cells of the MOB to the olfactory cortex.

CHAPTER II

INTEGRITY OF CHOLINERGIC AND GABAERGIC BASAL FOREBRAIN NEURONS IN AGING

Introduction

The basal forebrain refers to a boomerang shaped heterogeneous population of cells contiguously distributed beginning rostrally in the medial septum and extending caudally into the rostral portion of the lateral hypothalamic area (Sofroniew et al., 1987; Butcher and Woolf, 2004). Cholinergic and GABAergic neurons are co-distributed in these nuclei and comprise the majority of corticopetal BF neurons. Cholinergic neurons can be identified by labeling of either choline acetyl transferase (ChAT), the enzyme that joins Acetyl-CoA to choline to make ACh, or AChE. BF GABAergic neurons are on average two-fold more abundant than cholinergic neurons, but only a subset project to cortical targets (Rye et al., 1984; Saper, 1984; Kiss et al., 1990; Gritti et al., 1993). The BF GABAergic neurons do not form a homogenous population, but instead are classified as either interneurons that project to other BF neurons or projection neurons that innervate the hippocampus, olfactory bulb, or other cortical structures (Risold, 2004). GABAergic neurons in the rostral BF that project to telencephalic regions commonly express parvalbumin (PARV), a calcium binding protein that is associated with high cellular metabolism and a high cell firing rate (Gaykema and Zaborszky, 1997). Visualization of GABAergic BF neurons is

commonly achieved using antibody labeling of either PARV or the enzyme L-glutamic acid decarboxylase (GAD). GABAergic neurons use GAD to synthesis GABA, the main inhibitory neurotransmitter in the brain. In the BF, there are two isoforms of the GAD enzyme; GAD67 is found in the GABAergic projection neurons and GAD65 is found in the GABAergic interneurons (Castañeda et al., 2005).

In AD and the normal aging process, deficient cholinergic indices have been linked to reduced number and size of BF cholinergic neurons (Johnston et al., 1979; Whitehouse et al., 1982; Coyle et al., 1983; Mann et al., 1984; McGeer et al., 1984; Lowes-Hummel et al., 1989). Many studies to date have investigated cholinergic neuronal degeneration in aged rats using a number of detection methods, counting methodologies, strains, and ages (Gilad et al., 1987; Koh and Loy, 1988; Fischer, 1989; Altavista et al., 1990; Fischer et al., 1991; Armstrong et al., 1993; Lee et al., 1994; Smith and Booze, 1995; Stemmelin et al., 2000; McQuail et al., 2010). The large number of variables differing across studies makes it difficult to draw definitive conclusions as to the presence and magnitude of phenotypic and/or degenerative changes in cholinergic neurons during aging and, in particular, how such changes relate to cognitive dysfunction. For example, many early studies did not have the benefit of modern immunohistochemical detection methods and quantitative cell counting techniques such as semi-automated quantitative design-based stereology. This technique affords accurate estimates of total neuronal number

without assumptions regarding size, shape, or orientation of cells. Such methodology is critical for obtaining accurate estimates of the number of neurons, including those within BF, as age-related changes in size (both shrinkage and hypertrophy) of these neurons are well documented (Armstrong et al., 1993; Greferath et al., 2000; Veng et al., 2003; Smith et al., 2004).

It is becoming increasingly clear that corticopetal BF GABAergic neurons exert influences on neural transmission and cognitive functions linked to their terminal fields (Freund and Antal, 1988; Kiss et al., 1990; Freund and Meskenaite, 1992; Pang et al., 2001). GABAergic neurons are positioned to influence cortical circuitry, both through their direct input to cortical structures and via the interconnected cholinergic and GABAergic neural network within the BF (Freund and Antal, 1988; Freund and Meskenaite, 1992). Cholinergic BF neurons have been shown to provide extensive local collaterals to GABAergic BF interneurons and projection neurons. These GABAergic neurons reciprocally synapse on cholinergic neurons to create an interconnected neural system in the BF capable of providing feedback (Brauer et al., 1998; Zaborszky and Duque, 2000; Colom et al., 2005; Gritti et al., 2006). Surprisingly, relatively little is known regarding the anatomical integrity of the GABAergic projection neurons in AD (for review of existing literature, see McKinney and Jacksonville, 2005). In rats (F344), Smith and Booze (1995) reported no age-related loss of non-isoform specific GAD immunoreactive neurons. However, a reduction of PARV containing neurons has been previously reported in BF of aged rats (Miettinen et

al., 1993; Krzywkowski et al., 1995). Parvalbumin is a useful marker for identifying BF GABAergic projection neurons, as it is not expressed by GABAergic interneurons (Kiss et al., 1990; Gritti et al., 1993; Gritti et al., 2003). The functional impact of reduced PARV expression on local and cortical circuitry, and specifically how such changes might relate to age-related cognitive decline, remains unknown.

Methodologically sound, unbiased cell counts in the MS and both vertical limb of DB (vDB) and hDB are necessary to determine how aging affects GABAergic neuronal integrity and number, and if any changes are related to loss of cognition. Due to the extensive collaterals between the cholinergic and GABAergic BF systems, cholinergic cell counts in the same animals could contribute to understanding of how the two systems are affected in conjunction during the aging process and if changes are linked to a decline in cognitive function. In the present experiment we behaviorally characterized young and aged rats in the water maze to determine cognitive ability and subsequently performed confocal stereology on immunohistochemically labeled sections in order to estimate the number of cholinergic and GABAergic cells in the rostral BF. Estimated numbers of cholinergic and GABAergic projection neurons in BF were analyzed with regard to chronological age and cognitive status. We hypothesized that age-related changes in *both* cholinergic and GABAergic neurons contribute to declining mnemonic abilities at advanced ages.

Experimental design

Subjects

Young (6 mo; n=6) and aged (22 mo; n=12) male F344 rats, obtained from the National Institute of Aging colony, were behaviorally characterized on the Morris water maze (LaSarge et al., 2007; Bizon et al., 2009). Aged rats of 22 mo were used to investigate behavioral and neurobiological changes at advanced ages, as F344 rats average age of mortality is 24 - 26 mo (Lipman, 1996; Turturro et al., 1999; Harker and Whishaw, 2002; Nadon, 2006). Rats were housed in the Psychology Building at Texas A&M University for two weeks prior to the start of any behavioral testing. The AALAC- accredited vivarium was maintained at a consistent 25 degrees Celsius with a 12:12 hour light/dark cycle (lights on at 0800 hours). Rats had free access to food and water at all times. All rats in the study were screened for health problems including, but not limited to, cataracts, jaundice, food and water intake, and tumors. Sentinel rats, housed alongside the rats in this study, were routinely screened for a range of pathogens and found to be negative.

Morris Water Maze training

The Morris water maze task is widely used to assess spatial learning abilities in rodent models due to its sensitivity to damage to the medial temporal lobe (including hippocampus) and BF neurons (Morris, 1984; Sutherland et al., 1989). The maze consisted of a circular tank (diameter 183 cm, wall height 58

cm) painted white and filled with water (27° C) made opaque with the addition of nontoxic white tempra paint. The maze was surrounded by black curtains to which were affixed large white geometric designs that provided extramaze cues. A video camera mounted above the center of the maze was connected to a DVD recorder and computer, which were used for data storage and analysis using a video tracking system (Water 2020, HVS Image, UK). In the hidden platform task a retractable escape platform (diameter = 12 cm, HVS Image, UK) was submerged two centimeters below the water's surface in the southwest quadrant of the maze. In the cued platform task a black platform (diameter = 12 cm) that protruded 2 cm above the water's surface was located in a different quadrant on each trial.

Hidden platform training

Rats were trained as described in LaSarge et al. (2007). In brief, rats received three training trials a day for eight consecutive days. On each trial, rats were placed into the water facing the wall of the maze at one of four equally spaced start positions (north, south, east, or west). The start positions were varied in a pseudorandom fashion such that all rats started from each of the locations the same number of times. Rats were allowed to swim for up to 90 s in order to locate the platform before they were guided to it by the experimenter. Rats remained on the platform for 30 s, and subsequently were placed in a holding cage for a 30 s inter-trial interval. Every sixth trial was a probe trial, in

which the platform was lowered to the bottom of the maze for the first 30 s of the trial, after which it was raised to allow the rat to escape.

Visible (Cued) platform training

Following training on the reference memory task, rats were given a single session with six trials of cue training. In this session, rats were trained to escape to a visible platform that was moved to a different maze quadrant on each trial. Rats were given 90 s to reach the platform and were allowed to remain there briefly before a 30 s inter-trial interval. Visible platform training is not medial temporal lobe dependant, but has the same motivational and sensorimotor requirements as the hidden platform version allowing detection of deficits unrelated to memory that might contribute to performance deficits.

Statistics

Water maze data files were created by the Water 2020 software (HVS Image, UK). Data were imported to SPSS (v. 16.0) for analysis, and in all statistical comparisons described below, p values less than 0.05 were considered significant.

Visible (Cued) platform training

Pathlengths from visible platform swim trials 2 through 6 were averaged for individual rats; the first trial was excluded to control for acclimation to the

change from the hidden to the visible platform version of the water maze. Individual aged rats' pathlength averages were examined to confirm that they fell within 2 standard deviations of the young mean for inclusion in the larger data set. Group pathlength averages were compared using a one-factor ANOVA (pathlength average x age group).

Hidden platform training

Training trial data were averaged into four blocks consisting of the five trials preceding each probe trial, and performance was analyzed using cumulative search error. To calculate cumulative search error, the rat's distance from the platform location was sampled 10 times/s and these distances were averaged into 1 s bins. Cumulative search error is the sum of these 1 s bins minus the optimal path from the start location to the platform (Gallagher et al., 1993). Additional measures of performance (e.g., latency, swim speed) also were recorded. Data from interpolated probe trials (i.e. every sixth trial) were analyzed using mean search error. This measure was derived by dividing the cumulative search error by 30 s (i.e. the probe trial duration). Comparisons between age groups on both training trial blocks and probe trials were conducted using two-factor repeated measures ANOVAs (age X training trial block or probe trial) with Fisher's LSD post-hoc tests conducted where appropriate.

In addition to the comparisons described above, a Spatial Learning Index (SLI) score was derived for each rat using criteria described in Bizon et al. (2009). The SLI, first established by Gallagher et al. (1993), is calculated by weighting and summing mean search error from the interpolated probe trials to provide an overall measure of spatial learning ability for each rat. Weights for each probe trial were derived by dividing the mean search error in the young group on probe trial 1 by the mean search errors on probe trials 2-4. Using data from a large F344 study population, the weights assigned to each probe trial were: probe trial 2: 1.25; probe trial 3: 1.60; probe trial 4: 1.70 (Bizon et al., 2009). Lower SLI scores indicate better performance. A one-factor ANOVA and Fisher's LSD post hoc tests were used to assess differences between age groups using the SLI measure.

For group comparisons, aged rats were subgrouped based on SLI scores: aged-unimpaired (i.e. those aged rats that performed within the range of young rats) and aged-impaired (i.e. aged rats that performed outside this range). A two-factor repeated measures ANOVA (cognitive age group X probe trial) and Fisher's LSD post hoc analyses were used to compare training trial performance among these age groups.

Stereological estimates of neuron number

Tissue preparation

One week after behavioral training rats were rapidly euthanized with an

overdose of pentobarbital and perfused transcardially (0.9% saline followed by 4% paraformaldehyde). Brains were removed, postfixed in perfusate (24 hr) and cryoprotected in 20% sucrose in 0.1 M phosphate buffer (24 hr). Brains were sectioned through BF on a freezing microtome (coronal plane, 30 μm) and adjacent series of sections collected into cold cryoprotectant solution (25% glycerin. 25% ethylene glycol, 50% 0.1 M phosphate buffer, pH 7.4) and stored (–20^{o}C) until processing.

Immunofluorescence

Adjacent series of sections were processed for ChAT and GAD/PARV immunofluorescence to identify cholinergic and GABAergic BF projection neurons, respectively. Sections were rinsed in Tris-buffered saline (TBS; 100 mM Tris-HCl, 150 mM NaCl, pH 7.5), pre-incubated in blocking solution containing 3% normal donkey serum (NDS), 0.3% Triton X-100 in TBS for 1 hr and incubated in the blocking solution with the addition of the appropriate primary antibodies for 72 hr at 4˚C. Specific antibodies used were: goat anti-ChAT (Chemicon AB1449, 1:1000), rabbit anti-GAD67 (Bioworld, 1:500), and mouse anti-PARV (Sigma, P 3088, 1:500). After primary incubation, sections were rinsed in TBS, then incubated in TBS containing 2% NDS and appropriate fluorescent secondary antibodies (Alexa 488 donkey anti-rabbit, Alexa 555 donkey anti-goat, and Alexa 555 donkey anti-mouse; Molecular Probes, 1:300). Secondary incubations were performed for 2 h at room temperature in the dark

and sections were then rinsed in TBS and mounted onto Superfrost++ slides. Following immunohistochemistry, sections were coverslipped with ProLong Gold (Invitrogen), sealed with clear fingernail polish, and stored in the dark at 4^{o}C until analysis.

Quantitative stereology on a fluorescent confocal microscope (single- and multiple-labeling analysis)

Design-based stereological quantification was implemented on an Olympus Fluoview 300 confocal microscope, equipped with the appropriate filter sets, using a CCD camera and Stereo Investigator software. The Stereo Investigator software drove an X-, Y-, Z- Ludl motorized stage. Initial analyses determined the penetration of each antibody included in an individual experiment in order to restrict sampling in the Z-plane to those areas with complete labeling. The BF regions of interest were traced at low power magnification (4X), and the Stereo Investigator software was programmed to distribute equally-spaced sampling fields in the X, Y dimension within the outlined region. The BF regions of interest that were sampled, specifically the MS, vDB, and hDB, are shown in Figure 4 (as outlined in Paxinos and Watson, 1998). The software drove the stage such that sample areas were chosen in a systematic fashion. Neuron counting was performed at 40-60X magnification. Section thickness was measured at each sampling site. A guard zone of 3 μm at the top and bottom

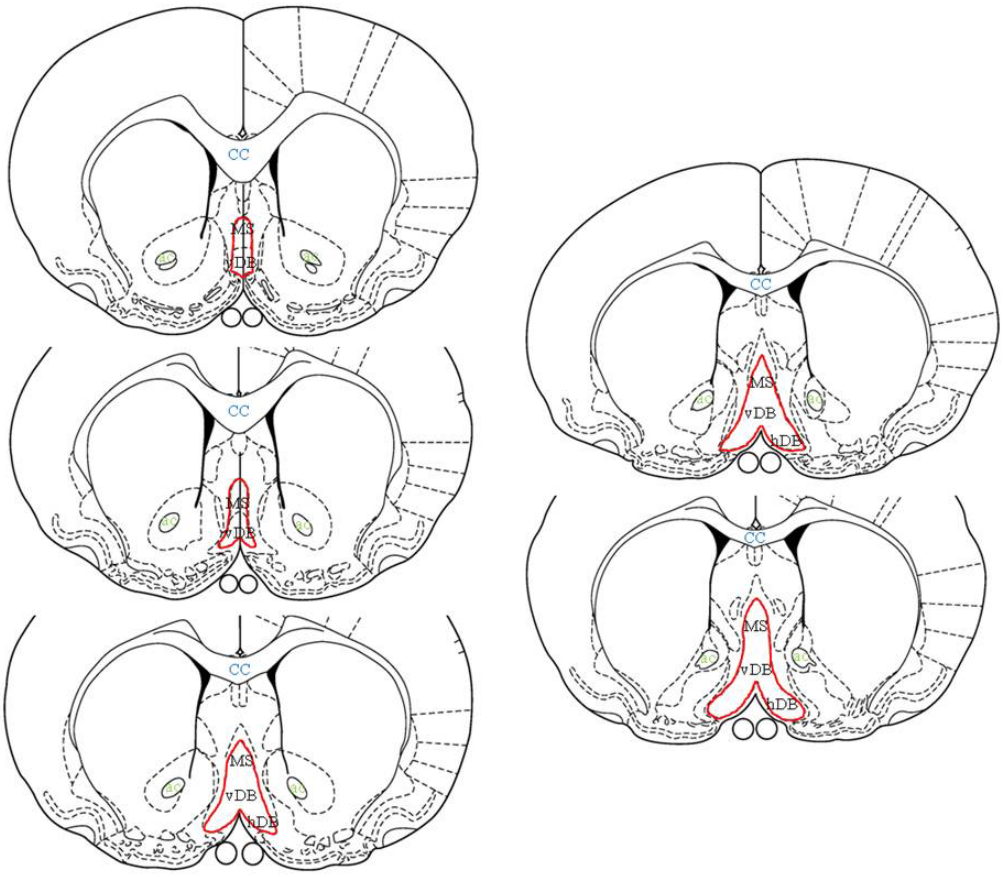

Figure 4. Anatomical regions and septohippocampal neuronal populations quantified. Medial septum, vertical Diagonal Band of Broca and horizontal Diagonal Band of Broca were traced as illustrated in figures 13-17 in Paxinos and Watson's The Rat Brain Atlas (red outline).

was implemented, in which no cells were counted. At each sampling site, Z-stacks (containing 1 μm Z-slices) of the entire counting frame were acquired serially at each wavelength corresponding to the labels of interest. Counting was then performed on the acquired Z-stacks allowing colors/labels to be visualized individually or simultaneously, aiding in accurate subcellular localization of multiple markers in the same cell (for GAD/PARV colocalized tissue). In each rat, cell counts were derived from approximately 10-12 sections, spaced at 120 μm intervals. These 10-12 sections spanned the entire rostral BF area, and were used to estimate total cell numbers in the region. The total number of single- or double-labeled neurons was estimated by multiplying the sum of each cell type counted by the reciprocal of the fraction of the BF nucleus that was sampled (i.e., the fraction of histological sections examined and the fraction of the total section thickness examined).

Statistical analysis of cell number

Estimates of BF neurons were calculated using the number of cells counted in the sampling sites and the area in the outlined region of interest for each animal. Stereology data files were created by Stereo Investigator (MicroBrightField, Wilmington, VT) and imported to SPSS (v. 16.0). In all statistical comparisons, p values less than 0.05 were considered significant. Total cell estimates of cholinergic neurons were derived from the number of ChAT+ neurons counted. In adjacent sections, total estimates of GABAergic

neuron number included: the total number of GAD+ neurons, the total number of PARV+ neurons, and the total number of GAD+ neurons that co-express PARV. Estimates of GABAergic or cholinergic BF neuron numbers, including cell estimates for populations labeled with each GABAergic markers, were compared as a function of age and cognitive abilities (i.e. water maze performance) using one-factor ANOVAs (cell estimate of phenotypic marker x age or cognitive ability). Additionally, individual estimates of GABAergic or cholinergic neurons and spatial learning ability were compared using a Pearson r correlation (bivariate correlation of SLI scores with estimates of total number of GAD, PARV, GAD/PARV, or ChAT cells).

Results

Cued (visible platform) task

Cued (visible platform) water maze was performed to control for sensorimotor and motivational deficits that might influence the performance of rats on the hidden platform water maze task. All aged rats performed visible platform training on par with young. A one-factor ANOVA revealed no differences in cued training between age groups ($F(1,23)= 0.23$, n.s.).

Spatial (hidden platform) trials

Training trials

Figure 5A shows the cumulative search error on training trial blocks for

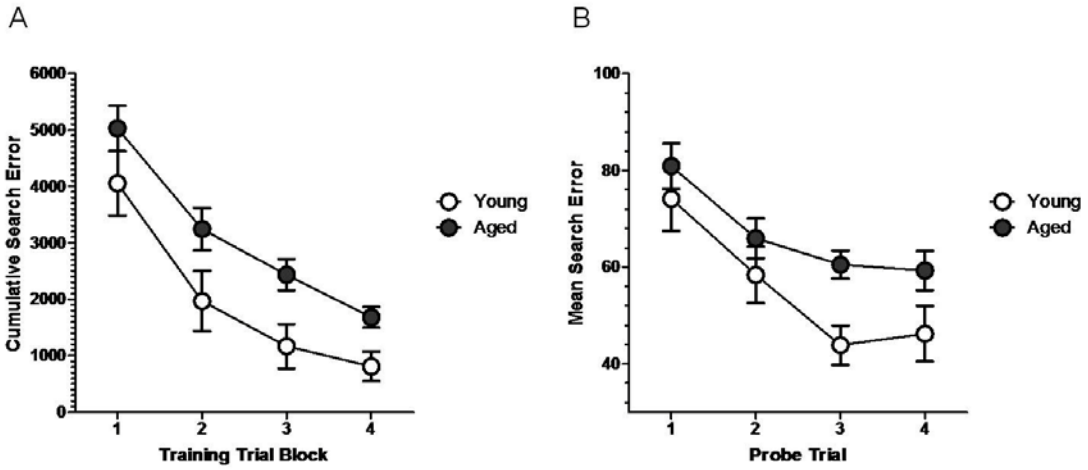

Figure 5. Training (A) and probe trial (B) data from young and aged animals during water maze training. Using both cumulative search error on training trials and mean search error on probe trials, all rats did learn, as demonstrated by the improvement in performance over the course of trials. However, aged rats were impaired as a group compared to young in both training and probe trials.

young and aged rats. A repeated measures ANOVA (age x training block) revealed that, although all rats improved in their search for the platform over the course of training ($F(3,66) = 39.86$, $p<0.01$), aged rats performed significantly worse as a group compared to the young rats across training trial blocks ($F(1,22) = 7.73$, $p<0.05$). The difference between age groups was not influenced by their initial abilities in the water maze, since a one-factor ANOVA (first training trial x age group) showed that young and aged rats did not differ in their search for the platform on the first training trial ($F(1,23) = 0.77$, n.s.).

Probe trials

Probe trial performance of young and aged rats confirmed training trial data. Figure 5B shows the mean proximity to the platform during the interpolated probe trials for young and aged rats. A repeated measures ANOVA (age x probe trial) revealed that all rats improved over the course of training ($F(3,66) = 12.90$, $p< 0.01$), but aged rats performed significantly worse than the young group ($F(1,22) = 7.71$, $p<0.05$).

Figure 6A shows individual performance of young and aged rats during probe trials, measured by their SLI score. For group comparisons, aged rats were subgrouped based on spatial learning performance using a SLI score cut off of 280, such that aged rats were classified as “aged-unimpaired” (< 280; n=6) or “aged-impaired” (> 280; n=6) (per Bizon et al., 2009). Figure 6B shows mean search error during probe trials separated by cognitive age group. Using

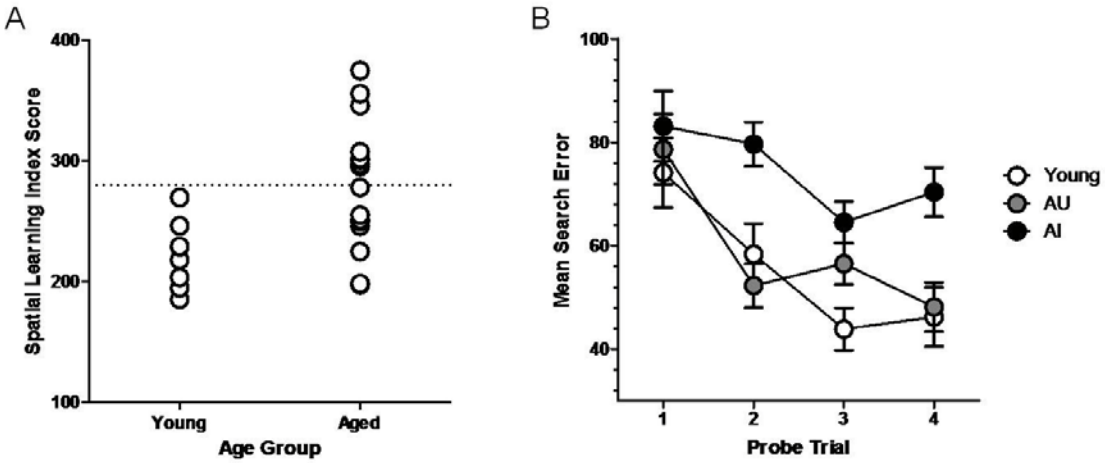

Figure 6. Spatial Learning Index scores (A) and probe trial performance of cognitive age groups (B). (A) Individual rats SLI scores (with higher scores indicative of worse performance) are marked by individual points and the upper range of young scores is marked by a dashed line. This graph demonstrated the individual variability in the aged group such that some perform on par with young (Aged unimpaired: AU) while other perform outside the range of the young cohort (above the line; Aged impaired: AI) demonstrating impairment. (B) Using the cognitive groups determined by the SLI scores on the left, the aged rat groups were split and graphed on their probe trial performance to show that the AU group did perform on par with the young and it was the AI group that was impaired in their performance in the water maze.

cognitive age group classifications, a repeated measures ANOVA (probe trial x cognitive age group) confirmed that all groups learned over the course of training ($F(3,63) = 13.67$, $p<0.01$), but there was a difference in performance between cognitive groups ($F(2,21) = 19.36$, $p<0.01$). Fisher LSD p*ost-hoc* analyses indicated that the aged-impaired group performed significantly worse during probe trials compared to both the young and aged-unimpaired groups ($p<0.05$), but there was no difference in performance between the young and aged-unimpaired groups.

Qualitative analysis of GAD and PARV expression in BF neurons

Figure 7 shows low and high power images of immunolabeling for GAD+, PARV+, and colabeled GAD/PARV+ neurons in MS/DB from a representative F344 rat. Immunostaining was uniform for all markers throughout the tissue independent of age and cognitive status, revealing full penetration of the antibodies. In agreement with previous findings, GAD+ and PARV+ neurons were codistributed throughout the MS/DB (for example Fischer et al., 1992; Gritti et al., 1993; Smith et al., 1993; Krzywkowski et al., 1995; Baskerville et al., 2006; Gritti et al., 2006). As seen in Figure 7, immunofluorescence with GAD and PARV antibodies resulted in a high degree of colocalization in both young and aged tissue (i.e. GAD/PARV+ cells). GAD+ and PARV+ cells ranged in shape from oval to polygonal, as previously described (Gritti et al., 2006).

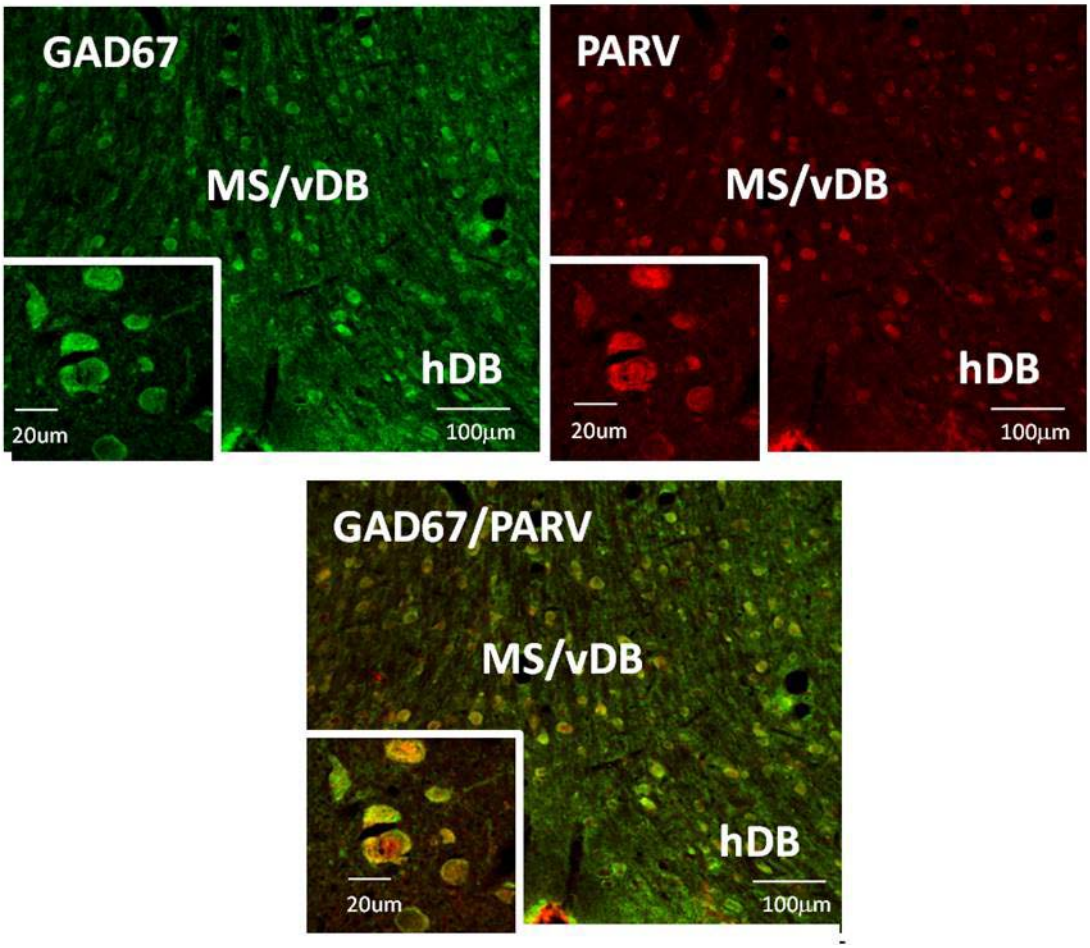

Figure 7. Low and high magnification images of GABAergic basal forebrain neurons. The medial septum (MS) and Diagonal Band of Broca (vDB) with GAD67 (488 laser, green), parvalbumin (555 laser, red), and the co-labeling of GAD67 and parvalbumin. Low power images, viewed with the 10x/0.36 objective, show the z-stack collected with a step size of 0.75 µm. High Magnification Images of GABAergic Basal Forebrain Neurons inset are shown from vertical limb of the Diagonal Band of Broca (vDB) with GAD67, parvalbumin, and the co-labeling of GAD67 and parvalbumin. High power images are viewed with a 60x/1.42 oil immersion objective, shown as the collapsed z-stack with a step size of 0.25 µm.

Stereological estimates of GABAergic neurons

Chronological age groups

Qualitative observations revealed no obvious loss of expression of GAD or PARV between age groups. Figure 8 shows the mean estimated GAD+, PARV+, and GAD/PARV+ cells derived by stereology in young and aged groups. The coefficient of error was ≤ 0.06 for all individual estimates. The coefficient of error is a number between zero and one that describes the accuracy of a stereological estimate, and the estimate is more reliable as the coefficient of error approaches zero. Although the aged group tended to have a greater number of labeled cells compared to young, one factor ANOVAs revealed no significant difference between young and aged rats in stereological estimates of GAD+, PARV+, and colabeled GAD/PARV+ cells (GAD+ ($F(1,17) = 2.43$, n.s.), PARV+ ($F(1,17) = 2.58$, n.s.), GAD+/PARV+ ($F(1,17) = 2.40$, n.s.)).

Cognitive age groups

Figure 9 and Table 1 show the mean stereological estimates of GAD+, PARV+, and colabeled GAD/PARV+ cell numbers (+/- SE) in young and aged rats subgrouped by spatial learning ability. With all GABAergic markers, an increase in number of immunoreactive cells was observed in aged rats with cognitive deficits compared to both young and aged-unimpaired rats. One-factor ANOVAs revealed a significant difference in the number of GAD+, PARV+, and

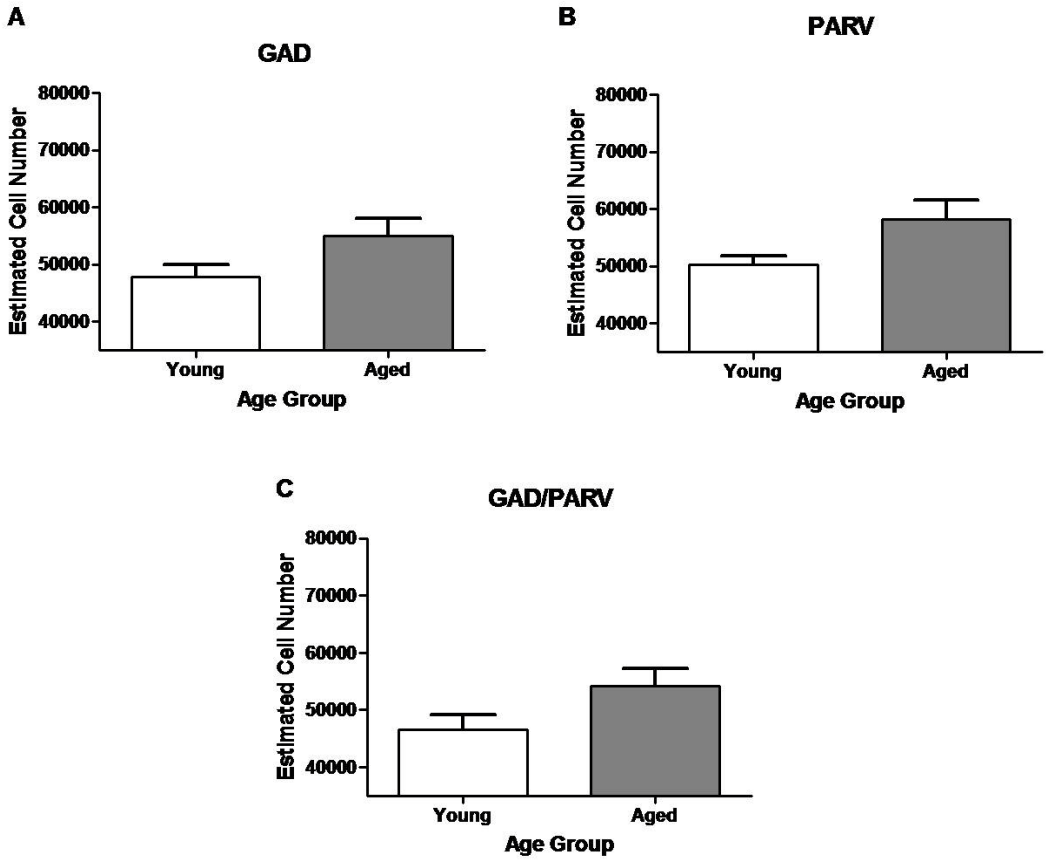

Figure 8. Estimated GAD+ (A), PARV+(B), and colabeled GAD/PARV+ (C) cell numbers in rostral basal forebrain for young and aged rats. GAD+ (A), PARV+ (B), and GAD/PARV+ colabeled neurons were stereologically estimated in young and aged F344 rats. There was no significant difference in cell numbers for any GABAergic phenotypic marker between young and aged rats.

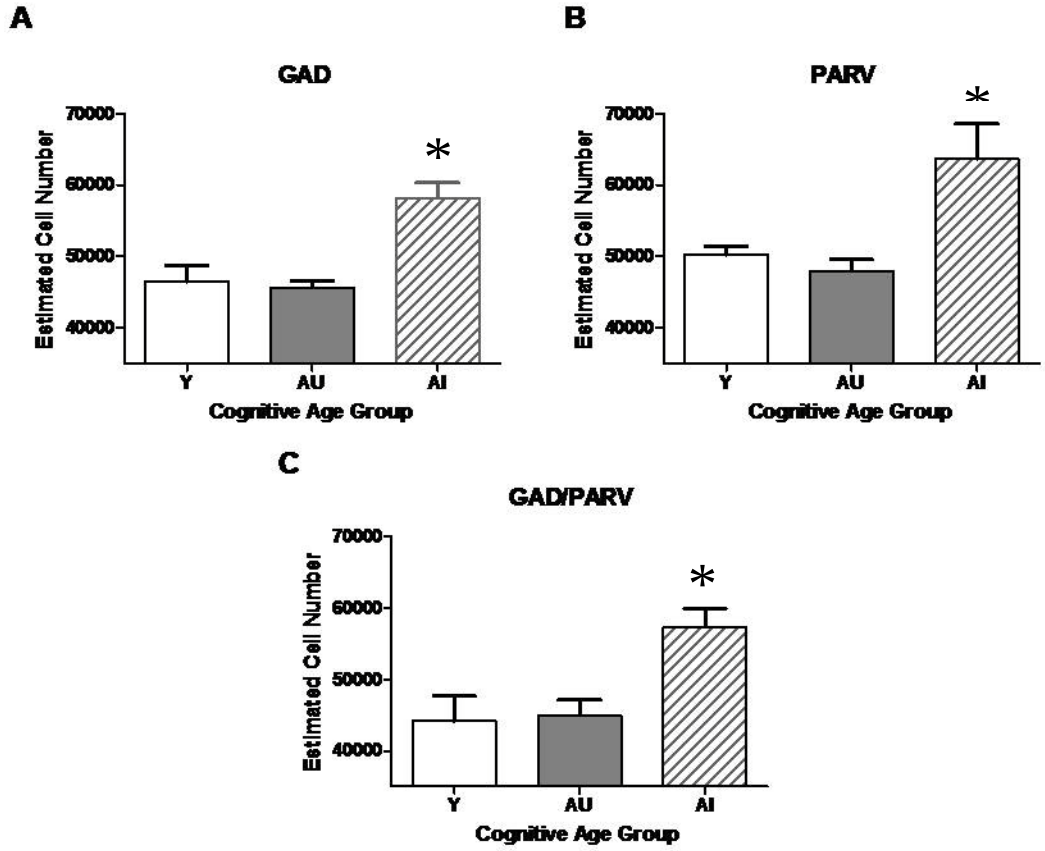

Figure 9. Estimated GAD+ (A), PARV+ (B), and colableled GAD/PARV+ (C) cell numbers in rostral basal forebrain for young, aged-unimpaired, and age-impaired rats. Aged rats were split into groups by their performance in the water maze and estimated cell numbers were determined using confocal stereology. The aged-impaired (AI) rats had an increased number of cells positive for GAD (A), PARV (B), and colocalized GAD/PARV cells (C) when compared to young or aged-unimpaired (AU) rats. (D) There was no difference in the estimated number of ChAT positive cells between cognitive age groups.

Table 1: Estimated Cell Population in Basal Forebrain by Cognitive Age

	GAD/PARV				
Cognitive Age	Mean	SEM	% change from young	% different from GAD	% different from PARV
young	46614.29	2576.64		-2.64	-7.30
unimpaired-aged	46148.19	1551.23	-1.00	-1.80	-6.60
impaired-aged	62181.81	3884.60	**33.40***	-1.39	-7.27

	GAD		
Cognitive Age	Mean	SEM	% change from young
young	47843.51	2056.26	
unimpaired-aged	46976.63	1119.01	-1.81
impaired-aged	63048.74	3723.52	**31.78***

	PARV		
Cognitive Age	Mean	SEM	% change from young
young	50283.61	1550.09	
unimpaired-aged	49406.91	1407.31	-1.74
impaired-aged	67055.64	4095.42	**33.35***

	ChAT		
Cognitive Age	Mean	SEM	% change from young
young	14535.95	1156.93	
unimpaired-aged	13645.15	207.64	-6.13
impaired-aged	13558.51	278.19	-6.72

GAD/PARV+ cells in the MS/DB between cognitive age groups (GAD: ($F(2,17) = 12.67$, $p<0.01$); PARV: ($F(2,17) = 14.03$, $p<0.01$); GAD/PARV: ($F(2,17) = 10.35$, $p<0.01$)). Fisher LSD post hoc analyses further revealed a significant increase of 32% in the number of GAD+ cells, a 33% increase in number of PARV+ cells, and a 33% increase in estimated GAD/PARV+ cells in the aged-impaired rats compared to young. Similar increases in cell numbers were seen in aged-impaired rats for all GABAergic markers when compared to aged-unimpaired rats ($p<0.01$). Post hoc analyses also showed that young and aged-unimpaired rats did not differ in their estimated cell number for any phenotypic marker.

Single labeled GAD+ and PARV+ populations

In young and aged rats, a small number of GAD+ and PARV+ cells did not colabel. The GAD+ only and PARV+ only cells were analyzed to determine any differences in their number associated with age or cognitive status. GAD+ only and PARV+ only cell numbers were determined by subtracting the number of co-labeled GAD/PARV+ cells from the GAD+ or PARV+ cells for each individual rat (i.e. GAD+ – GAD/PARV+ cells). T-tests (single labeled GAD+ or PARV+ cells x age group) confirmed that GAD+ only or PARV+ only cells did not differ between age groups ($t(16) = 0.42$ and 0.17, respectively; all n.s.). Additionally, one-way ANOVAs (GAD+ only or PARV+ only cells x cognitive status) revealed that neither GAD+ only ($F(2,17) = 0.08$, n.s.) nor PARV+ only ($F(2,17) = 0.19$, n.s.) cells differed as a function of cognitive status.

Relationship between GABAergic cell number and spatial reference memory performance

Figure 10 shows the relationship between individual water maze performance (SLI), and number of GAD+, PARV+, or GAD/PARV+ cells. Person correlation coefficients revealed a significant relationship between SLI scores and each marker of GABAergic projection neurons in aged rats: GAD+, $r = 0.68$; PARV+, $r = 0.76$; GAD+/PARV+, $r = 0.66$; all p's < 0.05; Table 2). In aged rats, worse performance in the water maze was associated with higher GABAergic cell numbers Similar relationships between SLI scores and GABAergic cell number (GAD+ and GAD/PARV+, but not PARV+) were observed among young rats with worse performance in the water maze associated with higher cell numbers. These correlations did not reach significance likely in part due to insufficient power (n = 6): GAD+, $r = 0.71$. p = 0.11; PARV+, $r = 0.15$, p = 0.78; GAD+/PARV+, $r = 0.80$, p = 0.06 (Table 2).

Qualitative analysis of ChAT expression in BF neurons

Sections adjacent to those used for estimating GABAergic cell numbers were processed for visualization of cholinergic neurons. Figure 11 shows representative ChAT+ immunostaining in MS/DB of young and aged tissue. ChAT+ cells were large, robustly labeled, and contained a well defined nucleus. In agreement with previous reports, ChAT+ cells had a polygonal and fusiform

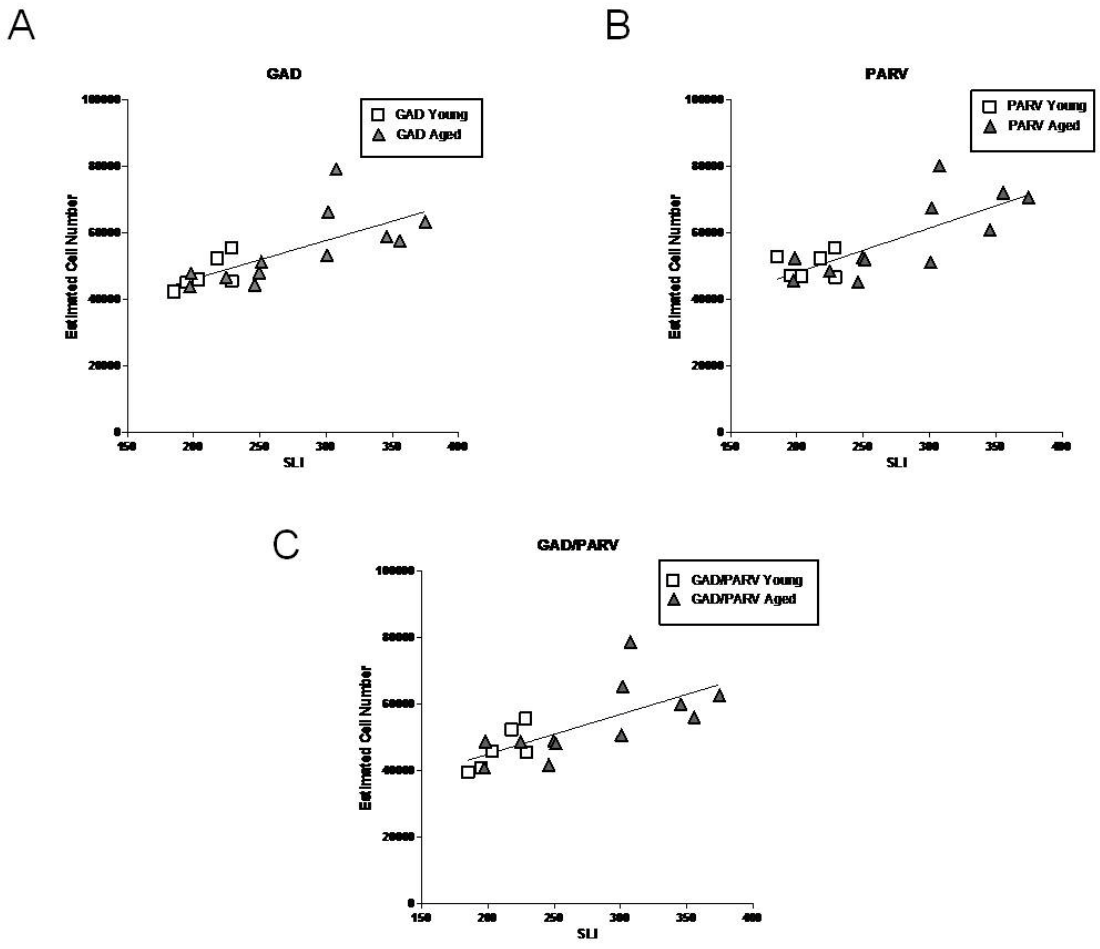

Figure 10. Correlational analysis of Spatial Learning Index Scores and estimated cell populations for GAD+ (A), PARV+ (B), and GAD/PARV+ (C) immunolabeled cells. Pearson's correlation coefficient revealed a relationship between GAD+, PARV+, and GAD/PARV+ cell numbers and water maze performance in aged rats, such that higher cell numbers were correlated with worse performance (higher SLI scores).

Table 2: Correlations of Estimated Cell Populations and Water Maze Performance

		YOUNG	AGED
		SLI	SLI
GAD	Pearson Correlation	.712	**.678**
	Sig. (2-tailed)	.112	**.015**
	N	6	**12**
PARV	Pearson Correlation	.151	**.760**
	Sig. (2-tailed)	.775	**.004**
	N	6	**12**
GadParv	Pearson Correlation	.800	**.661**
	Sig. (2-tailed)	.056	**.019**
	N	6	**12**
ChAT	Pearson Correlation	.457	-.017
	Sig. (2-tailed)	.362	.958
	N	6	12

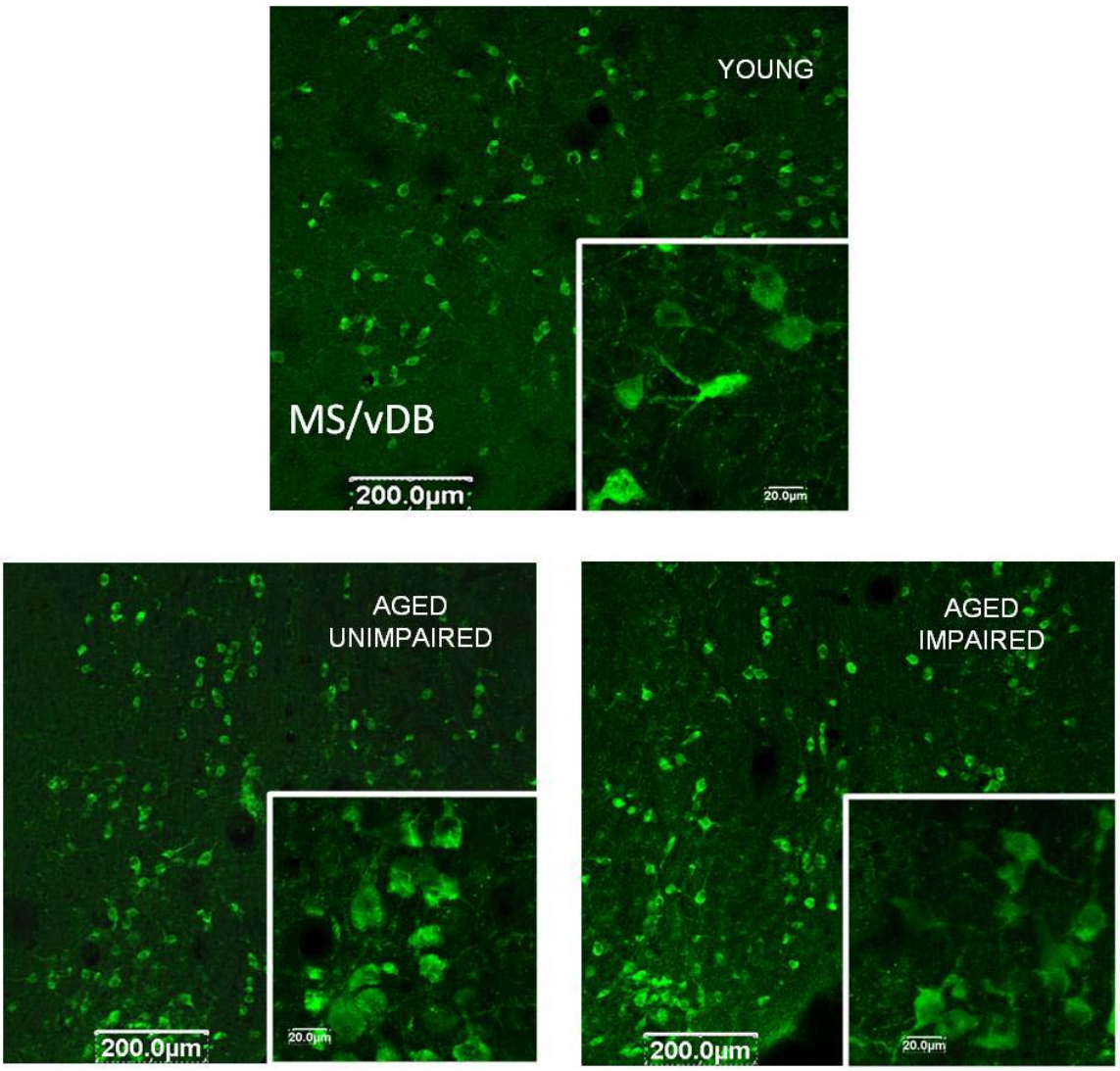

Figure 11. Low and high power images of cholinergic basal forebrain neurons in young, aged-unimpaired, and aged-impaired F344 rats. The medial septum (MS) and Diagonal Band of Broca (vDB) are labeled with ChAT (555 laser, shown in green). Low power images are viewed with the 10x/0.36 objective, and shown is the z-stack collected with a step size of 0.75 µm. A closer view of ChAT expression in cholinergic neurons in seen in the lower right corner. High power images, viewed with the 60x/1.42 oil immersion objective, show the collapsed z-stack with a step size of 0.25 µm.

shape (for example Gritti et al., 2006). Relative to GAD+ and PARV+ cells, ChAT+ cells were larger in size but smaller in number and less evenly distributed throughout BF nuclei (ChAT cells have a tendency to cluster). Analysis in the z-plane revealed full penetration of the ChAT antibody in both young and aged rats.

Cholinergic neuron number and age

Figure 12A shows the mean number of ChAT+ cells in young and aged rats. Stereological estimates of cell numbers showed that the number of ChAT+ neurons in rostral BF did not differ between young and aged rats. The coefficient of error was ≤ 0.07. A one-factor ANOVA revealed that the total number of ChAT cells in MS/DB was not significantly different between young and aged groups ($F(1,17)= 0.28$, n.s.).

Relationship between spatial reference memory performance and cholinergic neuron numbers

Figure 12B and Table 1 show the mean estimated ChAT+ cell number (+/- SE) for cognitive age groups. Analysis of ChAT cells between young, aged-unimpaired, and aged-impaired groups further confirmed that ChAT+ cell numbers remain consistent into advanced ages in F344 rats, regardless of cognitive status. A one-factor ANOVA showed no differences in ChAT+ cell numbers between young, aged-unimpaired, and aged-impaired groups,

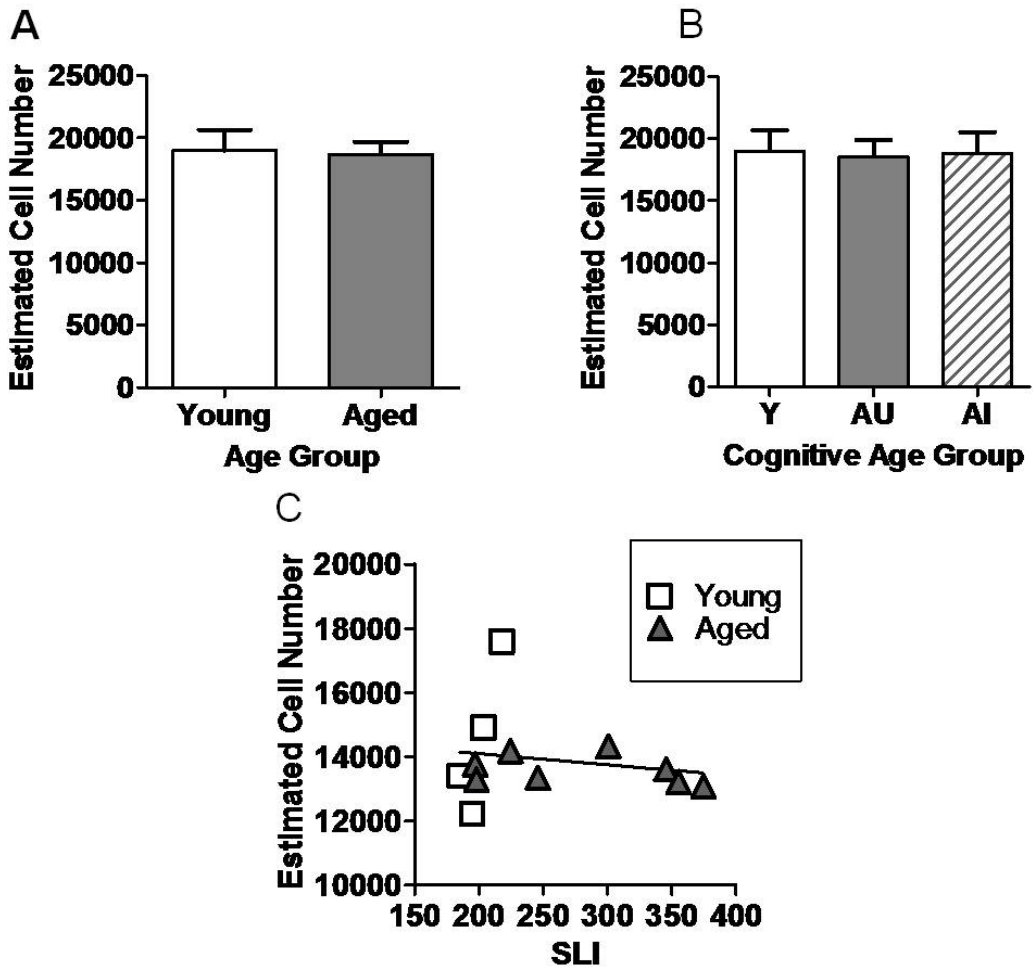

Figure 12. Estimated ChAT+ cells in the rostral basal forebrain for rats separated by chronological age (A) and cognitive age groups (B), and the correlation analysis of estimated ChAT+ immunolabeled cells and water maze performance (C). Aged rats were classified as Aged cognitively unimpaired (AU) and Age cognitively impaired (AI) by their performance in the water maze measured by Spatial Learning Index (SLI) score. Estimated cell numbers were determined using confocal stereology. There was no difference in the estimated number of ChAT positive cells between rats separated by chronological age (A) or cognitive ability (B). (C) Pearson's correlation coefficient revealed that ChAT+ cell numbers were not correlated with water maze performance.

regardless of water maze performance ($F(2,17)= 0.02$, n.s).

Figure 12C shows the relationship between individual rats' water maze performance (SLI) and ChAT+ cell number. Pearson's correlations coefficient revealed that spatial learning performance in the water maze was not correlated with ChAT+ cell numbers in young ($r = 0.46$, $p = 0.36$) or aged rats ($r = -0.02$, $p = 0.96$).

Discussion

Young and aged F344 rats were characterized in the Morris water maze in order to relate BF GABAergic and cholinergic neuron number to memory abilities. As previously shown, aged animals performed significantly worse as a group in the water maze compared to a young cohort in both training and probe trials (Gallagher et al., 1993; Bizon et al., 2009; for review see LaSarge and Nicolle, 2009). However, variability in spatial learning performance was observed such that some aged F344 rats performed on par with the young cohort, whereas others demonstrated impairment by performing outside the range of young. These differences in spatial learning performance were not due to motoric or motivational deficits, as performance on a cued (visible) version of the water maze did not differ between age and cognitive groups.

Tissue from young and aged behaviorally characterized animals was immunohistochemically labeled for identification of GABAergic (GAD67+ and PARV+) or cholinergic (ChAT+) projection neurons in the rostral BF. GAD67 is

the isoform of the GAD enzyme necessary to make GABA in GABAergic BF projection neurons (in contrast to GAD65 that is expressed by BF interneurons) (Castañeda et al., 2005). Expression of the calcium binding protein PARV in BF is also primarily restricted to the GABAergic projection neurons (Freund, 1989). As expected based on these previous observations (and the fact that these are two markers that should define the same population) GAD+ and PARV+ cells were highly colocalized throughout the BF areas quantified. Contrary to the hypothesis, we observed a marked increase in the number of GABAergic projection neurons selectively in aged-impaired rats compared to aged-unimpaired and young rats. No difference in GABAergic neuron number was detected in the aged-unimpaired when compared to young rats. This increase in number was detected by both immuno markers independently, as well as when the number of neurons co-expressing PARV and GAD67 were evaluated. Furthermore, a significant relationship was present between water maze performance and GABAergic cell numbers among aged rats, such that higher GABAergic BF projection neuron number was related to worse water maze performance. This relationship between worse cognition and higher GABAergic projection neuron number in BF was unique to the GABAergic neurons. Notably, in adjacent sections, cholinergic cell number was not significantly different between age or cognitive-age groups, nor was water maze performance correlated with cholinergic neuron number.

Age-related changes in GABAergic BF neuron number

Our data are consistent with previous findings that have described a resistance of BF GABAergic neurons to degeneration with advancing age. Using stereology, Smith and Booze (1995) estimated the number of non-isoform specific GAD+ neurons in BF of young and aged F344 rats using bright field microscopy. Although counts of GAD+ cells in this study were restricted to caudal BF (not considered in the current report), there was no difference in GAD+ cell estimates between young and aged rats. However, these results included GAD65+ neurons. If GAD65+ neurons (i.e., interneurons) declined with age, the decrease in number would mask any increase of GAD67+ neurons. Without use of stereology, Krzywkowski (1995) also reported no age-related changes in GABA+ cell counts in the same rostral areas of BF examined here (i.e. MS/DB). In the same study, Krzywkowski (1995) did observe an age-related reduction of PARV+ cells in MS/DB. Notably, young rats in the Krzywkowski study were 2-3 mo, and a reduction in PARV+ cell number was observed between 2 and 8 mo old rats. These data suggest that PARV is downregulated across the young adult lifespan and this may not have been detectable given the ages used in the current study (i.e. comparisons between 6 mo and 24 mo rats). It should be noted that Krzywkowski et al. (1995) also reported an increase in PARV+ cells between 15-16 mo and 26-27 mo, a finding supporting the current results in which elevated PARV+ cell number was detected in aged-impaired rats.

Age-related changes in cholinergic BF neuron number

Neither age nor cognition-associated changes were observed in estimates of the number of ChAT+ neurons quantified. The literature is inconsistent with respect to cholinergic cell degeneration with age, with some studies reporting cholinergic neuron loss and others failing to observe such reductions. In agreement with the current findings, several previous studies reported no age-related change in the number of cholinergic neurons between young and aged F344 rats (Gustilo et al., 1999; Ypsilanti et al., 2008). McQuail et al. (2010) also reported ChAT+ neuron number in MS/vDB did not change between young and aged F344 x Brown Norway hybrid rats. In contrast, Smith and Booze (1995) reported an age-related reduction in ChAT+ neuron number in F344 rats, although this study was restricted to caudal BF. Indeed, regional differences in cholinergic neurodegeneration may contribute to the inconsistency in reported age-related cell loss. Research in non human primates has shown a rostro-caudal gradient in the vulnerability of cholinergic neurons to age-related neurodegeneration in BF. Little to no loss of cholinergic cells is evident in rostral septal nuclei, but a significant age-related decline in cholinergic neuron number occurs in the more caudal nucleus (Stroessner-Johnson et al., 1992). Evidence of regional differences also has been found in the rat. One study in Sprague Dawley rats reported no reduction with age of ChAT+ neuron number in MS but a significant loss of these neurons in more caudal nuclei (de Lacalle et al., 1996). Regionally specific counts across distinct nuclei of the BF in a rostro-

caudal manner within the same animals would be very useful for reconciling the effects of age on cholinergic neuron number.

Although we found no change in the cholinergic neuron number with advancing age or cognitive decline, age-related changes in the cholinergic BF system may still contribute to age-related cognitive impairment. For example, changes in calcium homoeostasis have been reported in cholinergic neurons of rostral BF in aged-impaired F344 rats from the same study population of aged rats used in the current study (Murchison et al., 2009). Calcium is important for both the pre- and postsynaptic function, including the release of neurotransmitters, and a change in calcium homeostasis in cholinergic neurons may affect ACh release from the neuron. In addition to calcium dysfunction, there is evidence that aging may disrupt ACh signaling at the receptor level. An age-related decline in muscarinic ACh receptor function, as evidenced by blunted downstream g-protein signaling, has been reported and the degree of dysfunction was correlated with water maze performance (Zhang et al., 2007). Together with the current finding, these data suggest that although cholinergic cell number is stable with age, dysfunctional cholinergic signaling may contribute to age-related cognitive decline even in the absence of frank cell loss.

GABAergic and cholinergic BF cell counts in young rats

Previously estimates of BF GABAergic and cholinergic cells in young and aged rats have been derived using a variety of immunohistochemical counting

methodologies. Perhaps in part due to the differences in experimental procedures, previous stereological estimates of the total number of GABAergic and cholinergic cells in young rats have varied significantly. Differences in regional distinctions likely also contribute to discrepancies in cell counts between studies. There are not clearly defined nuclei in the BF, which leads to varied designations of these nuclei in anatomical regions of BF. Our GABAergic cell count reflects an increase from the upper range of a previous estimate that used roughly the same anatomical boundaries (Gritti et al., 2006: approximately 23,000 +/- 14,000 for MS/vDB and hDB). While the reasons for this shift upwards in total cell estimates is unclear, one possibility relates to progressive advances in the specificity of antibodies and/ or visualization of immunostaining. For example, GAD counts reported by the Gritti laboratory (1993, 2006) using identical parameters and anatomical boundaries increased from 30,000 to 119,000 across two studies conducted ten years apart. The lab attributed the increase to more specific labeling from the GAD antibody. Using roughly the same anatomical boundaries (MS, vDB, and hDB), our cholinergic cell count is consistent with a previous estimate of approximately 19,000 cholinergic neurons in the MS/DB (Miettinen et al., 2002). However, other stereological cholinergic cell counts of the same MS/DB region have been much lower (approximately 6,500 - 8,000) (Gritti et al., 1993; Gritti et al., 2006). Each of these cholinergic studies in addition to the current report employed different ChAT antibodies. Additionally, different immunohistochemical techniques also may impact cell

counts. Gritti et al. (1993, 2006) dehydrated their tissue for GABAergic and cholinergic cell counts before coverslipping. Dehydration causes significant tissue shrinkage, reducing section thickness by as much as half. In contrast, Miettinen et al. (2002) used a water-soluble epoxy resin embedding solution that functions similarly to the mounting media used here for fluorescent immunolabeling of GABAergic and cholinergic neurons; both minimize shrinkage in the three dimensional tissue structure. Miettinen et al. (2002) and the current study reported higher cholinergic cell counts compared to studies which used dehydration methods. Importantly, given identical parameters used within a study, experimental consistency allows for reliable between-group comparisons within a given study.

Implications of age-related changes in GABAergic neuron number for cognitive impairment

The selective increase in BF GABAergic projection neurons in the aged-impaired animals suggests that an increase in inhibitory signaling from BF to hippocampus might contribute to age-related memory loss. Indeed, a strong relationship was observed among aged rats such that higher numbers of GABAergic neurons were associated with worse memory performance. Evidence from pharmacology experiments has shown that increases in hippocampal GABA are associated with decreased performance in learning and memory. Hippocampal infusion of the GABA(B) agonist baclofen can impair

acquisition in water maze as evidenced by a larger search area for the platform (Arolfo et al., 1998). Notably, there is converging evidence that some well described actions of pharmacological agents targeting the cholinergic system may be mediated by GABAergic septohippocampal projections. Electrophysiological studies show that infusions of cholinergic antagonists atropine and scopolamine into the MS/DB, which also impair learning and memory, cause excitation of GABAergic septohippocampal projection neurons (Alreja et al., 2000; Wu et al., 2000). These data suggest that memory impairing properties of the cholinergic antagonists may be attributed at least in part to modulation of GABAergic projection neurons. Increases in GABAergic neuron number in BF may provide a means for cognitive impairment through an increase of inhibitory signaling to hippocampus in aged rats.

Age-related alterations in the hippocampal GABAergic system are pertinent to interpretation of the increase in GABAergic neuron number in BF described here. Age-related changes in hippocampal GABAergic cell number and protein levels have been described (Shi et al., 2004; Stanley and Shetty, 2004; Segovia et al., 2006; Gaviln et al., 2007). Life span studies have shown a decrease in GABAergic cells in hippocampus, from adult to middle age, with a further decrease from middle age to advanced ages (Vela et al., 2003; Stanley and Shetty, 2004; Gaviln et al., 2007). These decreases in hippocampal GABAergic neuron number included interneurons, to which BF GABAergic cells project, Interestingly, although the percentage of GABAergic neurons in

hippocampus was reduced, there was no age-related reduction in overall neuron number, indicating that this population of GABAergic neurons may exhibit a loss of phenotype but not actually degenerate (Shi et al., 2004; Stanley and Shetty, 2004). Despite the significant loss of hippocampal GABAergic neurons that often occurs with advancing ages, it has been reported that basal levels of GABA in the hippocampus measured via microdialysis remain consistent between 2 mo and 25 mo (Segovia et al., 2006). Thus, increases in GABAergic signaling from BF may compensate for the loss of hippocampal GABAergic neurons and help to maintain GABA levels in hippocampus.

Identification of new GABAergic neurons in aged-impaired rats

Although there is an increase in GABAergic BF neurons number in aged-impaired rats, there is no evidence that neurogenesis occurs in the BF (for review Riddle and Lichtenwalner, 2007). If the increased GABAergic neuron number is not a result of new neurons, another BF neural population must be contributing to this increase in GABAergic neuron. Interestingly, many neurons in the septohippocampal system have been shown to exhibit multiple phenotypes, including both GABAergic and glutamatergic, GABAergic and cholinergic, and cholinergic and glutamatergic (Manns et al., 2001; Colom et al., 2005; Gritti et al., 2006). Retrograde tracers combined with immunohistochemistry were used to determine that 25% of septohippocampal projection neurons are glutamatergic, but over 45% of these neurons also

express another phenotypic marker for either GABAergic or cholinergic cells (Colom et al., 2005). Another stereology study reported that over 90% of neurons in MS and over 89% in the DB were glutamatergic; this glutamatergic population colabeled with 36.0% of MS and 20.6% of DB GABAergic neurons and 4.4% of MS and 14.5% of DB cholinergic neurons (Gritti et al., 2006). These data suggest that BF neurons have the capacity to express multiple neurotransmitters, and make it intriguing to speculate that the increase in GABAergic projection neurons observed in aged-impaired rats may be specific to a neuron population that expresses (or did express) another phenotype. Due to the morphology and consistent cell number estimates of cholinergic septohippocampal neurons, it seems unlikely that the cholinergic system would be involved in the elevation of GABAergic markers. However, the smaller and more abundant GABAergic neurons do have similarities to the glutamatergic neurons in the BF. Glutamatergic neurons that project to the hippocampus are an average of 15 µm in diameter, which is similar to GABAergic septohippocampal projections neurons (on average 14.5 µm in diameter) (Colom et al., 2005). Additionally, glutamate is a precursor to GABA, the formulation of which requires GAD enzyme-induced decarboxylation of glutamate. Future studies examining the number of neurons with glutamatergic phenotypic markers throughout the lifespan and colocalization with GABAergic phenotypic markers should be of great value in determining if the age-related GABAergic increase is due to neural shift of phenotype.

Conclusion

In conclusion, young and aged rats were trained in the water maze, and while a subgroup of aged rats performed on par with young, the other aged rats were impaired compared to young. Confocal stereology was used to determine GABAergic and cholinergic cell numbers in young and aged rats. GABAergic neurons were selectively increased in aged rats with impaired water maze performance, compared to young and aged-unimpaired rats. Moreover, water maze performance was correlated with GABAergic BF neuron number in aged rats, in which rats with worse performance had higher GABAergic neuron numbers. Cholinergic cell number remained consistent between young and aged rats and was not correlated with water maze performance. Further studies focused on determining the cell population in which the GABAergic markers are upregulated, as well determining the functionality of the GABAergic BF neurons in this aged cognitively impaired group will be useful for understanding the functional implications of these findings. Additionally, the upregulation of GABAergic septohippocampal projection neurons in aged-impaired rats suggests that pharmacologically targeting the GABAergic system may be an effective alternative to the traditional AChE therapies to treat age-related cognitive deficits and should be further explored.

CHAPTER III

PHARMACOLOGICAL REVERSAL OF AGE-RELATED COGNITIVE DECLINE WITH A GABA(B) ANTAGONIST*

Introduction

Basal forebrain cholinergic and GABAergic projection neurons are well-positioned to directly impact mnemonic function in hippocampus and other medial temporal lobe structures, the functions of which are sensitive to precipitous decline in aging (e.g., explicit/declarative and spatial learning and memory; Frotscher and Leranth, 1986; Freund and Antal, 1988; Baxter et al., 1996; Pang and Nocera, 1999). As such, these transmitter systems are logical targets for therapies to improve cognitive capacities in aging. Drugs that enhance cholinergic activity (either through direct agonistic actions at cholinergic receptors or by increasing ACh availability by inhibiting AChE activity) enhance a variety of cognitive functions across species (for review, please see Parent and Baxter, 2004) and most currently available treatments for age-related cognitive decline are AChE inhibitors (Fischer, 1989; Smith and Booze, 1995; Gibbs, 1998; Gilmor et al., 1999; Doggrell and Evans, 2003; Jones, 2003; Parent and Baxter, 2004). Indeed, patients with mild cognitive impairment (MCI), or loss of cognitive function including memory loss without a pathological disease, that

take AChE inhibitors show increased hippocampal activity and improved performance on explicit memory tasks, demonstrating that these drugs do offer clinical benefit (e.g.,Gron et al., 2006). However, this therapeutic avenue in isolation has limitations, as the cognitive enhancing effects of AChE inhibitors in aged individuals are transient; after 3 years, MCI patients with and without AChE inhibitor treatment are cognitively equivalent (Petersen and Morris, 2005). Moreover, this class of drugs only appears effective in improving mild loss of cognitive functions and offers little benefit to aged individuals with moderate to severe learning and memory deficits (Kaduszkiewicz et al., 2005; Birks and Flicker, 2006; Pelosi et al., 2006; Raschetti et al., 2007).

In humans, olfactory functions are increasingly being recognized as being vulnerable to age, and olfactory identification and discrimination deficits have been linked to more troubling dysfunction in other types of cognition such as declarative memory processes mediated by the medial temporal lobe (Gabrieli, 1996; Freedman et al., 2002; Eibenstein et al., 2005; Wilson, 2006). In agreement with these results, our group recently reported such a relationship in aged F344 rats (LaSarge et al., 2007). As observed among humans, considerable variability naturally occurs among the aged F344 rat population such that some aged rats maintain cognitive abilities on par with young cohorts while others develop marked and significant cognitive impairment with advancing age (Bizon et al., 2009). We observed that the same sub-population of aged F344 rats that demonstrates impaired spatial reference memory in the

Morris water maze was also impaired in the ability to discriminate odors despite the fact that these rats had comparable odor detection abilities and could discriminate among other sensory stimuli as well as young and aged-unimpaired cohorts (LaSarge et al., 2007).

Notably, in our previous study, odor discrimination learning abilities in individual aged F344 rats were highly consistent across novel odor discrimination pairs. Aged rats classified as “learning-impaired” eventually reached criterion levels of performance on a given discrimination problem, but there appeared to be neither savings of prior learning rules nor a practice effect across subsequently presented odor pairs. Indeed, these rats were just as impaired on their third odor discrimination problem as on their first. As such, the olfactory discrimination task presented itself as particularly well-suited for assessing the ability of pharmacological agents to improve age-related cognitive impairment. First, the task is as effective as the Morris water maze for identifying learning-impaired rats within the aged F344 study population. Second, the reliability of the olfactory discrimination learning deficit in aged rats allows for the use of a within-subject experimental design in which performance of each subject can be evaluated with and without drug treatment.

Data from lesion studies in young subjects indicate that coordinated actions of cholinergic and GABAergic signaling are critical to many aspects of cognition affected by age (Baxter et al., 1995; Pang and Nocera, 1999; Pang et al., 2001; Parent and Baxter, 2004; Yoder and Pang, 2005), and therefore drug therapies targeting the

GABAergic system may offer novel but complementary treatment avenues for dementia. Specifically, antagonists at the GABA(B) receptor appear to be promising candidates, as compounds from this drug class reportedly enhance cognitive function across a wide range of tasks and species in young subjects (Mondadori et al., 1996a; Mondadori et al., 1996b; Flood et al., 1998; Getova and Bowery, 1998; Escher and Mittleman, 2004; Froestl et al., 2004; Helm et al., 2005; Berta et al., 2009). For example, in young rodents and non-human primates, the most well-studied GABA(B) receptor antagonist, SGS742 (CGP36742), improves performance in a two-way active avoidance task and spatial reference memory in the eight-arm radial and Morris water mazes (Getova and Bowery, 2001; Froestl et al., 2002; Helm et al., 2005; Chan et al., 2006). In the Helm et al. (2005) study, improved memory was associated with decreased hippocampal CREB2 (ATF4) activity, indicating one site of action and possible mechanism following systemic administration of this compound (Vernon et al., 2001; Chen et al., 2003; Helm et al., 2005). The clinical utility of this class of pharmaceuticals is further supported by the wide range of effective doses at which enhanced learning and memory is observed in young subjects and few side effects associated with the efficacious doses (Blake et al., 1993; Mondadori et al., 1996a; Mondadori et al., 1996b; Getova and Bowery, 2001; Helm et al., 2005; Chan et al., 2006; Emson et al., 2007). Nevertheless, surprisingly few studies have examined GABA(B) antagonists as a possible treatment for age-related cognitive dysfunction. In this study, the GABA(B) antagonist CGP55845 was assessed for its ability to improve odor discrimination learning deficits in aged F344 rats. We expected that administration of CGP55845 prior to testing would result in fewer trials- and errors-to-criterion (indicating improved performance) compared to saline vehicle in aged cognitively-

impaired rats, based on other studies investigating the effects of GABA(B) antagonists on cognitive function in both animals and humans (Mondadori et al., 1993; Getova and Bowery, 1998; Getova and Bowery, 2001; Froestl et al., 2004; Helm et al., 2005).

Experimental design

Young adult (6 mo, n=10) and aged (22 mo, n=17) male F344 rats obtained from the National Institute on Aging colony (Harlan, IN, USA) were individually housed in the AALAC-accredited Psychology Department vivarium at Texas A&M University with a regular 12:12h light/dark cycle (lights on 08:00) and climate control at 25 °C. Rats were given free access to food and water except during discrimination testing, when they were food-restricted to 80% of their free-feeding weights. All rats were screened daily for health problems including but not limited to cataracts, jaundice, food and water intake, and the appearance of tumors. Sentinel rats housed in the same room were further screened for a range of pathogens, and all blood work was negative throughout testing. All animal procedures were conducted in accordance with approved institutional animal care procedures and NIH guidelines.

Olfactory discrimination learning was tested according to LaSarge et al., (2007). Briefly, the test apparatus consisted of an opaque plastic box (49 x 33 x 28 cm) divided by an opaque Plexiglas barrier into holding (16 cm) and test (33 cm) compartments, the latter of which contained two terra cotta flower pots arranged side-by-side against the rear wall. Behavior was scored by an

experimenter blind to drug treatment using a video feed to a TV monitor that allowed the rats to be viewed through the rear wall of the test compartment.

Initially, rats were shaped to dig for a food reward (1/4 of a Froot Loop, Kellog's, Battle Creek, MI) buried at varying depths in the pots, which were filled with home cage bedding (wood shavings). Raising the Plexiglas barrier marked the start of each trial and rats were considered shaped to dig when they successfully obtained the food reward buried 2 cm below the surface of both pots in under 2 minutes.

For discrimination problems, pots were filled with clean home cage bedding and the rims of the pots were scented with two different odorants (e.g., rose+ and citrus-). Odorants used were perfume oils obtained from The Bath Junkie and The Body Shop and 10 µl of the full strength oil was applied to pots. Novel odors were used for each discrimination problem (i.e. each odor was used only once). Only one pot contained the food reward (+), and the odor of the food was disguised by crushed Froot Loops sprinkled over the bedding filling both pots. The position (left or right) of the rewarded pot was varied pseudo-randomly across trials. Criterion performance on each problem consisted of six consecutive trials in which the correct (baited) pot was chosen. Rats were considered shaped to discriminate after reaching criterion performance on two odor problems prior to the onset of pharmacological testing.

Pharmacological testing began on a separate day after completion of shaping. Young and aged rats received i.p. injections of one of three doses of

the GABA(B) receptor antagonist CGP55845 (0.001, 0.01 or 0.1 mg/kg; Tocris, Ballwin, MO) or 0.9% saline vehicle alone (1 ml/kg) 40 min prior to testing. The number of trials to reach criterion was used as the measure of performance. The order of injections was as follows: CGP55845, saline, CGP55845, saline, CGP55845, saline, with the order of presentation of the doses of CGP55845 randomized across rats and age groups. Only one dose of CGP55845 or saline was given each day and a 48 hour washout period was interposed between injections (during which no testing was conducted). Rats were tested in two cohorts; rats in the first cohort were tested using the 0.1 and 0.01 mg/kg doses (and saline). A second cohort of animals was tested with these two doses and an additional 0.001 mg/kg dose. This dose was added to provide a more comprehensive dose response curve after it was clear in the initial cohort that *both* 0.1 mg/kg and 0.01 mg/kg doses significantly improved performance.

After completion of discrimination testing, a subset of rats was tested for their ability to detect and respond to decreasing concentrations of odorants following vehicle (saline) and the highest effective dose of CGP55845 (0.1 mg/kg). Significant attrition due to the length of time necessary to complete odor discrimination testing and test odor detection abilities with and without drug resulted in only a subset of subjects (N=5 young adult; N= 4 aged learning-unimpaired and N=3 aged learning-impaired) completing this testing. Only animals that completed all testing, including the doses of CGP 55845, washout, saline, and odor detection assessment with and without drug were included in

this latter analysis. For these tests, rats were trained on two new odor discrimination problems as described above (one for saline and one for CGP5585) using novel full strength odorants paired with mineral oil (unscented pot). For both problems, the novel odor was rewarded. After reaching criterion performance on the new problem, rats were assessed for their ability to respond to decreasing concentrations of the same odorant (diluted 1:10, 1:100, or 1:1000 in mineral oil) versus mineral oil alone. Assessment of performance at descending dilutions was performed immediately after reaching criterion on the initial problem. Rats received 16 trials at each dilution and the percentage of correct responses was used to assess performance. Saline and CGP 55845 odor detection testing were performed on different days with at least a 48 hour interval between assessments.

Results

In agreement with our previous report (LaSarge et al., 2007), significantly greater variance in performance was observed among aged rats compared to young adult rats following vehicle injections (Levine's Test of Equality of Variance performed on mean trials to criterion on saline problems: $F_{(1,28)}=6.55$, $p<0.05$) with some aged rats performing on par with young cohorts (hereon referred to as aged-unimpaired rats) and others demonstrating marked and consistent impairment across multiple discrimination problems (hereon referred to as aged-impaired rats). As shown in Figure 13, young adult rats (N= 10)

averaged 10.15 +/- 3.22 (S.D.) trials-to-criterion on saline problems. Performance of each aged-unimpaired rat (N=11) fell no more than one standard deviation above young adult performance (mean =8.26 +/- 1.89 S.D.; animals under line in Figure 13). All other aged rats were classified as aged-impaired (mean trials to criterion = 19.35 +/- 4.40 S.D.; N=9; animals above line on Figure 13).

All rats received odor discrimination sessions following saline and two doses of CGP55845 (0.1 mg/kg and 0.01 mg/kg). As shown in Figure 14, although aged-impaired rats needed more trials to reach criterion after saline compared to young adult and aged-unimpaired rats, after both doses of the GABA(B) antagonist, aged-impaired rats performed on par with the other two groups. These observations were confirmed using a two-factor repeated measures ANOVA (Cognitive Age Group X Drug Condition). The ANOVA revealed main effects of Cognitive Age Group ($F_{(2,57)}$= 7.34, $p < .01$) and Drug Condition ($F_{(2,54)}$= 3.25, $p < .05$), as well as an interaction between Cognitive Age Group and Drug Condition, such that the drug effect on performance

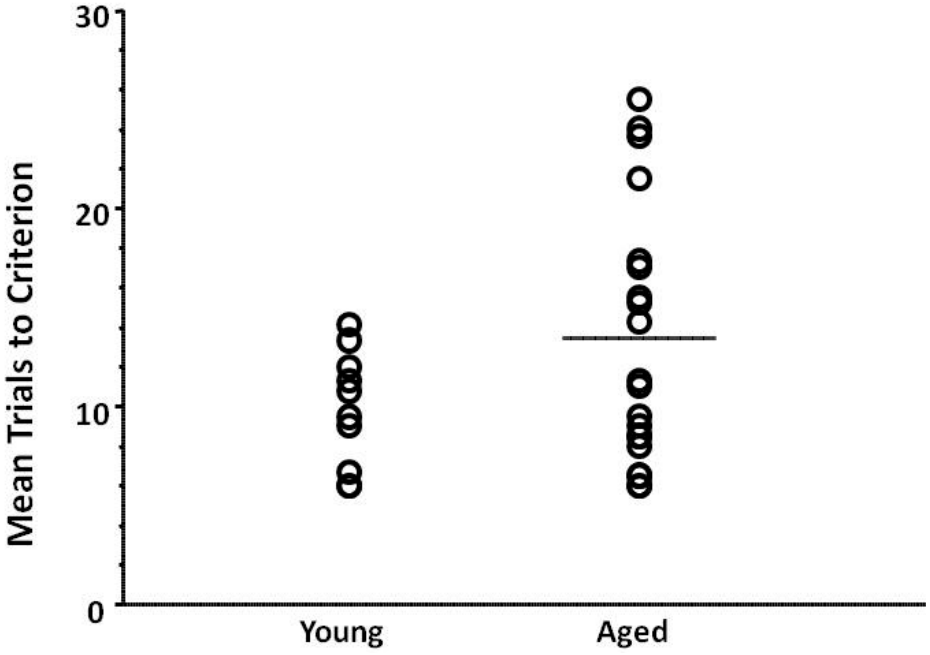

Figure 13. Mean trials to criterion of individual young and aged rats across saline odor discrimination sessions. Black line indicates the classification of aged subjects into aged-unimpaired and aged-impaired groups. All aged-impaired rats fell outside the young mean + S.D., while all aged-unimpaired rats performed within that criterion.

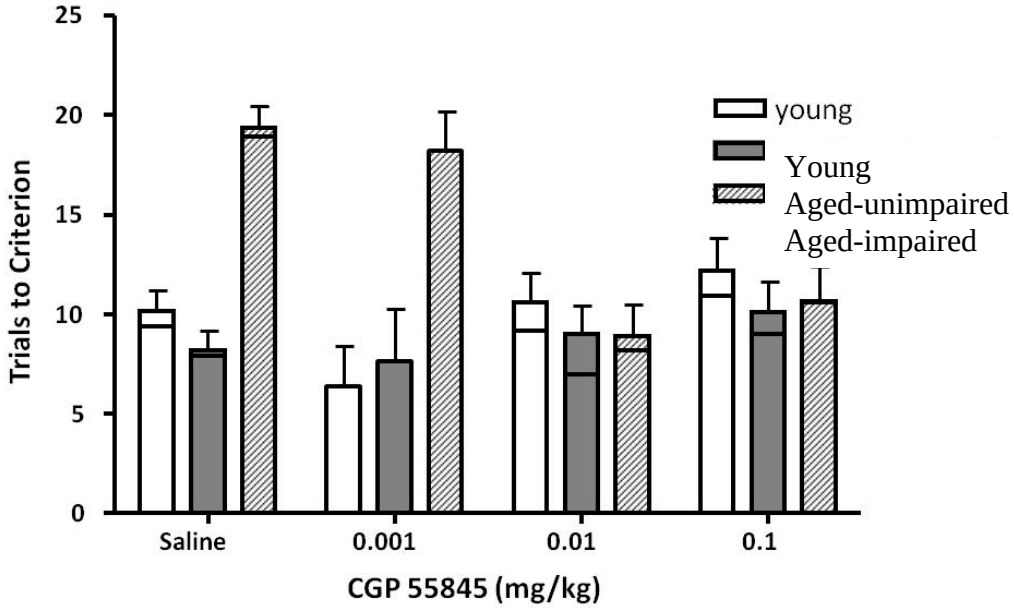

Figure 14. Mean (+/- S.D.) odor discrimination performance after administration of the GABA(B) antagonist CGP55845 or saline vehicle in young, aged-unimpaired, and aged-impaired rats. There was a significant Cognitive Age Group by Drug Condition interaction such that treatment with 0.01 and 0.1 mg/kg of CGP558445 improved trials to criterion specifically in the aged-impaired group. Black lines indicate means in the subset of rats tested with the 0.001 mg/kg dose, which as shown was ineffective in reversing the learning deficit in the aged-impaired group.

differed across Cognitive Age Groups ($F_{(4,54)}$= 5.22, p < .01). To confirm that the interaction was a result of the drug improving the performance (trials to criterion) of aged-impaired rats, a series of one-factor repeated measures ANOVAs within each Cognitive Age Group with Drug Condition as the sole within-subjects factor was performed. There was a main effect of drug condition in the aged-impaired group ($F(_{2,16})$= 11.73, p<0.01) but not the young or aged-unimpaired groups (Fs<0.58, n.s.). *Post hoc* pair-wise comparisons confirmed that the aged-impaired group performed significantly better with both the 0.01 and 0.1 mg/kg dose of CGP 55845 when compared to their saline control trials (all p's<0.05).

Given that both doses of CGP55845 initially tested were effective in reversing the learning deficit in aged-impaired rats, additional testing was performed with a lower dose of CGP55845 (0.001 mg/kg) to better define the minimum effective dose of this drug. Only a subset of animals was tested at this dose (n=5 young adult; n=3 aged-unimpaired; n=5 aged-impaired). These animals received all three doses of CGP55845 and saline in the randomized order as described above. A repeated measures ANOVA (Cognitive Age Group X Drug Condition) was performed on only those subjects that received all three doses of CGP55845 to test efficacy of the lower dose. Although no main effect of drug was observed ($F_{(3, 30)}$ = 1.22, n.s.), the ANOVA did reveal a main effect of Cognitive Age Group ($F_{(2,10)}$= 11.96, p <.01) and a significant interaction between Cognitive Age Group and Drug Condition ($F_{(6, 30)}$ = 2.41, p =.05). To confirm that the interaction was consistent with the larger dataset and that it

resulted from the drug improving the performance (trials to criterion) of aged-impaired rats, a series of one-factor repeated measures ANOVAs was conducted within each Cognitive Age Group with Drug Condition as the within-subjects factor. There was a main effect of drug condition in the aged-impaired group ($F_{(3,12)}$= 3.80, $p<0.05$) but not the young or aged-unimpaired groups (Fs<0.998, n.s.). *Post hoc* pair-wise comparisons showed that even with the small group size there was still a significant improvement in performance of aged-impaired rats with the 0.01 mg/kg dose of CGP 55845 when compared to saline ($p<0.05$); the additional lower dose of 0.001 mg/kg was not effective.

We have previously found no evidence for olfactory detection deficits in aged learning-impaired F344 rats (LaSarge et al., 2007). Nevertheless, it is possible that CGP55845 enhanced olfactory discrimination learning in this subgroup by enhancing olfactory detection abilities. Hence, after completion of discrimination testing, subsets of rats from each Cognitive Age Group (n=5 young adult; n=4 aged-unimpaired and n=3 aged-impaired) were tested for their ability to detect and respond to decreasing concentrations of odorants following vehicle and the highest effective dose of CGP55845 (0.1 mg/kg). As shown in Figure 15A and in agreement with the above data, on the discriminations performed prior to odor detection testing, a two-way ANOVA of Cognitive Age Group and Drug Condition revealed main effects of Cognitive Age Group ($F_{(2, 8)}$ = 5.77, $p <.01$) and Drug Condition ($F_{(1, 8)}$ = 7.18, $p <.01$), and an interaction between Cognitive Age Group and Drug Condition ($F_{(2, 8)}$ = 12.29,

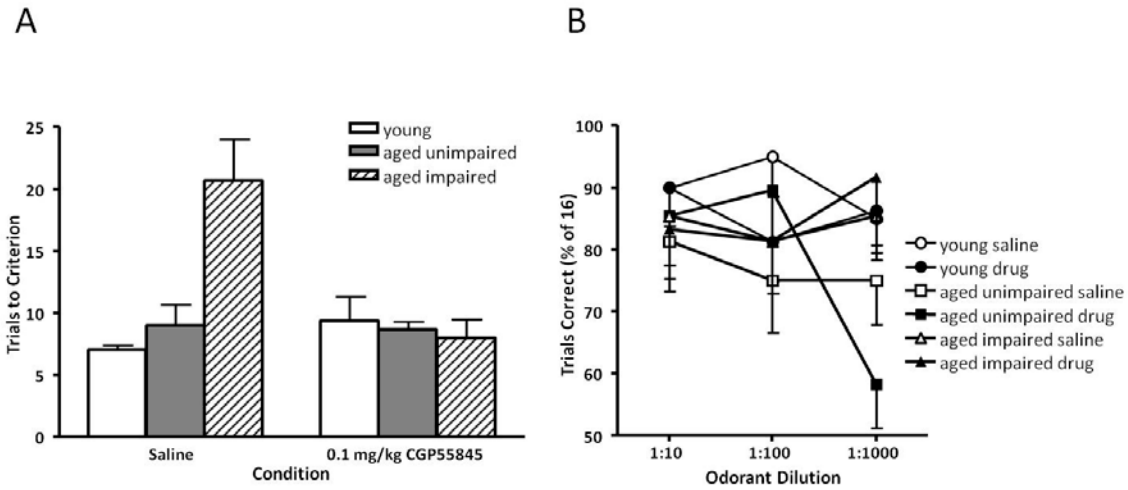

Figure 15. Olfactory detection testing with and without GABA(B) antagonist. (A.) Mean (+/- S.D.) odor discrimination performance in young adult (open bar), aged-unimpaired (solid grey bar), and aged- impaired (hatched grey bar) rats after injection with either saline (control condition) or 0.1 mg/kg CGP55845 (highest effective dose shown in Fig. 1) on discrimination problems prior to odor detection testing. The aged-impaired group performed significantly worse than both young and aged-unimpaired under the control condition (saline), an impairment that was once again reversed under 0.1mg/kg CGP 55845. (B.) After all rats reached criterion performance on the odor discrimination problems with saline or CGP55845, the rats were assessed for their ability to detect odors at decreasing dilutions. There were no differences between Cognitive Age Groups in their ability to detect an odorant at 1:10, 1:100, or even a 1:1000 dilution of the full strength odor.

p <.01). Fisher's PLSD post hoc analyses confirmed that aged-impaired rats performed significantly worse than young adult and aged-unimpaired rats in acquiring the problem after saline (p's< 0.05 in both cases) but not on the problem acquired after CGP55845. Figure 15B shows the percent accuracy on the two discrimination problems (saline and 0.1 CGP55845) on which the odor predictive of the reward was progressively diluted (1:10, 1:100 and 1:1000 of the full strength odor). A repeated measures ANOVA revealed no main effects or interactions involving Drug Condition, suggesting that the drug did not influence odor detection abilities. In addition, a planned comparison of performance on descending dilutions of only the aged learning-impaired rats (the group in which performance was selectively enhanced by CGP55845) after saline and CGP55845 also revealed no main effects or interaction, including at the 1:1000 dilution ($F_{(1, 5)} = 0.45$, n.s.), suggesting that the improved olfactory discrimination learning observed following CGP55845 in this study was not likely a result of the drug altering gross olfactory abilities.

Discussion

Results from the current study are the first to demonstrate that acute treatment with the GABA(B) antagonist CGP55845 can completely ameliorate robust learning discrimination deficits that reliably occur in a subset of aged F344 rats. Notably, this enhancement of learning performance was selective to aged-impaired rats as performance of young and aged-unimpaired cohorts was

not significantly affected by CGP55845 at any dose. Moreover, the reversal of learning deficits by CGP55845 in aged-impaired rats did not appear to be a result of the drug influencing gross olfactory detection ability, as all cognitive age groups performed similarly at a 1000-fold dilution of the odorants used for drug testing with or without the presence of the drug.

In addition to providing support for the use of this GABA(B) antagonist as a treatment for age-related learning and memory impairment, these data also demonstrate the utility of the simultaneous two-choice odor discrimination task in the F344 aging model for preclinical assessment of pharmacological interventions to improve learning deficits in aging. Reliably, approximately half of the aged subjects are cognitively impaired in this naturalistic aging model, with the same subset of aged subjects demonstrating marked deficits assessed in the Morris water maze and olfactory discrimination tasks (LaSarge et al., 2007; Bizon et al., 2009). In agreement with our findings, Robitsek et al. (2008) recently reported that a subset of aged rats impaired in the water maze task also performed poorly in an odor recall task, further implicating a role of hippocampus in odor learning. Unlike in the water maze, the ability to alternate vehicle (no drug) sessions with drug sessions and to observe a consistent return to baseline performance in the absence of drug within individual subjects even after numerous (upwards of 10) novel discrimination problems allows for stringent interpretation that the drug is responsible for changes in learning performance.

Indeed, when using novel odors, there appears to be little to no savings from learning of prior odor discrimination problems.

The relationship between performance on the two tasks (water maze and the simultaneous two-choice odor discrimination task) in aged rats does suggest that the septohippocampal pathway critical for spatial learning may also be involved in the odor discrimination task used here. Previously, Eichenbaum et al. (1988) found that lesions of the fimbria fornix, which result in deafferentation of both cholinergic and GABAergic projections from BF to hippocampus, impair learning in a similar simultaneous two-choice odor discrimination task (Eichenbaum et al., 1988; Eichenbaum et al., 1989). A number of studies have identified the hippocampus as a common site of action following systemic administration of GABA(B) antagonists, through mechanisms such as disinhibition of excitatory presynaptic terminals and direct actions on post-synaptic hippocampal neurons (Kulik et al., 2003). GABA(B) receptor activation in GABAergic pre-synaptic terminals causes a decrease in Ca2+ conductance which inhibits neurotransmitter release, such that blockade of these receptors has a net effect of enhancing hippocampal signaling. Post-synaptic activation of hippocampal GABA(B) receptors increases K+ conductance and CREB2 activation, a transcription factor implicated as a regulator of memory suppressor genes (White et al., 2000; Bettler et al., 2004; Helm et al., 2005; Emson et al., 2007). In support of the involvement of such a mechanism in the effects observed in the present study, Helm et al. (2005) showed that systemic

administration of the GABA(B) antagonist SGS742 both facilitated memory and suppressed hippocampal CREB2 activation through its interaction with CRE, reducing basal CRE binding in young rats. While involvement of the hippocampus is suggested, there is also evidence that lesions of the dorsal striatum, and not hippocampus, disrupt simultaneous two-choice odor discrimination learning (Jonasson et al., 2004; Broadbent et al., 2007). The use of systemic administration allows for the drug to affect performance through multiple anatomical areas that, although involved in performance, may not be critical for odor learning.

The absence of memory facilitation in young subjects in the current study in comparison to Helm et al. (2005) could be reflective of the different drugs used but is more likely due to differences in task difficulty between the two studies. In the current study, young and aged-unimpaired rats performed proficiently in the two-choice odor discrimination task, producing very few errors in the absence of the drug. Thus, there was very little parametric space in which to observe an enhancement of performance in these groups. Notably, there was a modest trend toward enhancement of learning in young rats at the lowest dose (which was ineffective for aged-impaired rats) and performance in young and aged-unimpaired rats was numerically worse at the highest dose of the drug tested here (0.1 mg/kg) relative to saline performance, suggesting that in young subjects too much blockade of GABA(B) receptors may impair performance. Together with the Helm study, these data suggest that there may be an optimal

level of signaling via the GABA(B) receptor that affords maximally proficient cognitive performance in both young and aged subjects.

CHAPTER IV

DISCUSSION AND CONCLUSION

Introduction

With the average lifespan of humans increasing each decade, the population over the age of 65 years is growing steadily (Xu et al., 2010). This increase in the number of individuals at advanced ages has resulted in an elevation in the frequency of age-related disorders, with both Alzheimer's disease (AD) and Parkinson's disease in the top 15 reasons for mortality (Xu et al., 2010). Age-related impairment is not an inevitable consequence of the aging process. Variability in the population exists in which some humans show decline in declarative memory tasks in their fourth decade and others maintain performance well into advanced age (Albert, 1993; Small, 2001; Bizon and Nicolle, 2006).

The individual variability inherent to cognitive aging in humans has been modeled in the F344 rats. In accordance with previous studies, data presented in Chapter II showed that a subpopulation of F344 rats succumbs to cognitive decline at advanced ages while other aged rats maintain cognitive function on par with young subjects (LaSarge et al., 2007; Bizon et al., 2009). The variability among aged rats observed in this model allows age-related changes in neurobiology to be related to not only the chronological aging process but also to cognitive aging.

It has been suggested that neuroanatomical changes (e.g. cell size, number, and phenotypic markers) in BF cholinergic and, to a lesser degree, GABAergic systems may contribute to age-related cognitive disorders. Previous research on the cognitive decline in AD showed a reduction in ChAT expression in AD patients compared to age matched controls (Kuhar, 1976), and loss of cholinergic BF neurons in patients with AD, particularly in the nucleus basalis (Whitehouse et al., 1982). These findings led Bartus et al. (1982) to propose the cholinergic hypothesis, stating that loss of cholinergic function is associated with, and possibly causal to, cognitive decline and AD. The cholinergic hypothesis of AD has been used to further explain cognitive deficits related to both natural aging and disease states. As a result, there has been extensive characterization of the cholinergic system over the past 30 years, including age-related changes. In contrast, data on the neuroanatomical characteristics of the GABAergic BF system in aging is much more limited, and almost absent with respect to age-related changes associated with cognitive decline.

Interactions of basal forebrain cholinergic-GABAergic systems: Implications for cognition

Elevation in hippocampal ACh levels have been linked to learning and memory. Microdialysis experiments showed hippocampal extracellular ACh increases during hippocampal dependent learning and memory tasks (for example Fadda et al., 1996; Yamamuro et al., 1996; Fadda et al., 2000;

McIntyre et al., 2002). Systemic pharmacological treatments that impair memory decrease hippocampal extracellular ACh, and drug treatments that enhance memory have been shown to increase ACh in the hippocampus (Parent and Baxter, 2004). Additionally, GABA released in the BF inhibits the synthesis and release of ACh from BF cholinergic afferents in hippocampus. Intraseptal infusions of the GABA(A) agonist muscimol resulted in reduced hippocampal ACh activity and also impaired memory (e.g. spatial memory) (Brioni et al., 1990; Durkin, 1992). In contrast, blockade of GABA(A) receptors in BF via microinfusions of bicuculine results in increased hippocampal ChAT activity and facilitation of hippocampal based learning and memory (Kenigsberg et al., 1998).

Although there is a plethora of data to support the hypothesis that age related alterations in the BF cholinergic system accompany cognitive decline, it is equally clear that this projection system is not solely responsible for age-related memory loss. Fornix transections that disconnect the hippocampus from BF produced impaired hippocampal based learning and memory, including in the water maze (Morris, 1982). However, selective lesions of either the cholinergic or GABAergic BF projection neurons alone did not replicate the learning deficit observed following fornix lesions (Baxter et al., 1995; Pang et al., 2001). Importantly, coordinated disruption of both cholinergic and GABAergic systems in concert did produce significant impairment in spatial learning and memory, although notably, combined lesions of cholinergic and GABAergic neurons still did not produce the extent of damage in spatial learning and memory induced by

fornix transections (Morris, 1982; Pang et al., 2001). Furthermore, learning impairments produced by either the muscarinic ACh receptor antagonist scopolamine or a GABA(A) receptor agonist muscimol are enhanced by lesions of the cholinergic system, suggesting that these compounds are acting on GABAergic septohippocampal cells (Pang and Nocera, 1999). Together, these data show that the age-related changes in the cholinergic system are not solely responsible for deficits that emerge in learning and memory, and suggest that GABAergic projection neurons have an equally important role in cognitive functions supported by the hippocampus. Furthermore, age-related changes in the GABAergic system, in concert with the cholinergic system, might contribute to cognitive loss.

Role of GABAergic system in age-related cognitive impairment

In Chapter II, age-related cognitive impairment was associated with an upregulation of GABAergic cells in rostral BF. Aged F344 rats behaviorally characterized in the water maze were impaired compared to young, but further analysis indicated that only a subset of the aged rats were in fact impaired. Through the use of immunofluorescence and confocal stereology, GABAergic cells (i.e. cells containing GAD67 and/ or PARV) were visualized and counted in young and aged rats. Surprisingly, aged-impaired rats had an elevation GABAergic neuron numbers of over 30%, across all markers, compared to both young and aged-unimpaired rats. Moreover, higher numbers of GABAergic BF

projection neurons were associated with greater memory impairment among aged rats, suggesting that changes in the GABAergic septohippocampal system contribute to age-related cognitive impairment.

It is possible that the increased number of GABAergic septohippocampal neurons observed in aged-impaired rats may reflect a compensatory mechanism initiated by age-related alterations of the hippocampal GABAergic system. Previous studies reported a loss of GABAergic hippocampal interneurons with age (Shi et al., 2004; Stanley and Shetty, 2004; Gaviln et al., 2007). Although decreases in GABAergic hippocampal interneurons have been detected in aging, suggesting a decrease in hippocampal GABA levels, a microdialysis study showed that aged rats have the same basal levels of GABA in the hippocampus as young rats (Segovia et al., 2006). This stability in hippocampal GABA levels across ages, even after hippocampal GABAergic interneuron degeneration, may indicate the presence of a compensatory mechanism whereby additional GABA is released from terminals projecting to hippocampus from other regions, such as BF. Moreover, an age-related increase in GABAergic neuron number may be one mechanism whereby optimal levels of GABA are maintained in hippocampus.

Changes in the BF GABAergic system can affect hippocampal pyramidal cell activation. GABAergic septohippocampal neurons synapse onto hippocampal GABAergic interneurons that in turn project to pyramidal cells. Thus, GABAergic signaling from BF results in a net disinhibition on hippocampal

pyramidal cells. Similarly, cholinergic BF cells release ACh and induce a subsequent depolarization of the hippocampal pyramidal cells. This complex interaction between cholinergic and GABAergic systems allows the two systems to work together to control whether pyramidal cells become de/hyper-polarized. If increases in hippocampal GABA results from additional BF afferents that synapse on GABAergic interneurons, these septohippocampal neurons could facilitate an environment in which less inhibitory signaling from hippocampal interneurons prevents hyperpolarization of the hippocampal pyramidal cells. However, the disinhibition could create a situation where even blunted ACh signaling from BF could lead to the excitation of the pyramidal cells. Additionally, due to the loss in hippocampal GABAergic interneurons, there may already be less inhibitory signal at the hippocampal pyramidal cells. It is possible that the increase in GABAergic phenotypic markers are in BF neurons of another phenotype, like newly characterized glutamatergic septohippocampal neurons that project to pyramidal cells (see Figure 1) (Huh et al., 2010). If the additional GABAergic markers are in BF septohippocampal neurons of another phenotype, and those cells are functional (i.e. release GABA), it may lead to an overall increase in inhibitory tone in the hippocampus. Furthermore, intrahippocampal infusions of GABA agonists have been shown to impair learning and memory, and therefore it is likely that release of GABA by septohippocampal cells would impede learning and memory processes (for review Izquierdo et al., 1991; Farr et al., 2000). Understanding the BF cell population in which the upregulation of

GABAergic markers is present in those animals with age-related cognitive impairment could help to determine future pharmacological therapies to reverse cognitive dysfunction.

Role of cholinergic system in age-related cognitive impairment

Although cholinergic BF neurons in adjacent tissue sections remained consistent in number between young and aged rats (Chapter II), given other findings, it is likely that reduced cholinergic signaling with age may be a contributing factor to impaired cognition. Indeed, both nicotinic and muscarinic ACh receptors are reportedly vulnerable to age. Uchida et al. (2000) found an age-related reduction in nicotinic receptors throughout the brain, and others have reported decreased expression and binding of Ach to the M2 subtype of muscarinic (Narang, 1995). Furthermore, blunted signaling via muscarinic receptors has been observed in aged rats impaired on water maze (Zhang et al., 2007). Other age-related cellular changes in cholinergic neurons may further contribute to attenuated cholinergic signaling in aging. Aged-impaired F344 rats from the same study population of behaviorally-characterized F344 rats used here have increased calcium cell buffering levels in rostral BF cholinergic cells relative to young and aged-unimpaired rats. One interpretation of this increased buffering is that it may reflect calcium dysregulation in cholinergic neurons of aged-impaired rats. The functional implications of this finding could include disrupted ACh signaling from BF to the hippocampal pyramidal cells (Murchison

et al., 2009). Disruption of ACh signaling from BF, combined with age-related changes in the cholinergic receptors, may reduce ACh signaling in hippocampus and facilitate an age-related decrease in cognitive function.

Role of septohippocampal system in olfactory learning

In Chapter III, a GABA(B) receptor antagonist was shown to reverse olfactory learning deficits selectively in aged-impaired rats. These finding have clear implications for the GABAergic system in age-related cognitive decline which is described in more detail below. On a more fundamental level, these data are suggestive of a role for GABAergic systems in olfactory learning. Notably, this role is not entirely clear from the data generated in our studies and remains a fertile ground for future research. Although the GABA(B) antagonist was able to improve odor discrimination performance in aged-impaired rats, performance was not significantly altered in age-unimpaired and young rats The young rats, however, did have a trend towards enhancement of learning at the lowest dose of CGP 55845. Importantly, a floor effect may have prevented the detection of drug effects on olfactory discrimination learning in young and aged-unimpaired rats. In the simultaneous odor discrimination task, criterion was met at 6 correct trials in a row. Young and aged-unimpaired rats needed very few trials over this criterion to complete the task with saline administration compared to the aged-impaired, and the performance of the young and aged-unimpaired rats left little room for improvement (averages for trials to criterion were

approximately 10 for young, 8 for aged-unimpaired, and 19 for aged-impaired). Using a more difficult olfactory task in future experiments help clarify whether CGP 55845 or other pharmacological agents targeting the GABAergic system can influence learning performance in young subjects.

Insights regarding the role of GABAergic systems, and specifically basal forebrain GABAergic systems in olfactory discrimination learning can be garnered from our understanding of neuroanatomical circuitry involved in different types of olfactory learning. While the MOB is responsible for the transduction of odorants, downstream circuitry implicated in the ability to correctly associate odors to reward and to discriminate between two odors is distinct, depending on the specific demands of the task (for example Eichenbaum et al., 1988; Eichenbaum, 1998; Setlow et al., 2003; Illig, 2005; Schoenbaum et al., 2006). Specifically, the neural systems implicated in odor learning appear to depend upon whether odors are presented simultaneously or in succession (sequential). Successive odor discrimination problems are facilitated by fornix and EC lesions and therefore do not appear to be mediated by the hippocampus / MTL (Eichenbaum et al., 1988; Otto and Eichenbaum, 1992). Instead, neural recording during go/no-go odor successive discrimination problems show OFC and ventral striatum are involved in this type of cue predictive odor learning (Setlow et al., 2003). The two-choice odor discrimination learning task used in this dissertation includes simultaneous odor presentation (two odors are presented together with only one associated with a reward).

Interestingly, other labs have reported impaired performance resulting from fornix lesion (as well as lesions of the EC and perirhinal cortex) on a two-choice (simultaneously presented) odor discrimination task similar to that used in our studies (Eichenbaum et al., 1988; Eichenbaum et al., 1989; Otto and Eichenbaum, 1992; Otto and Garruto, 1997; Alvarez et al., 2002). In addition to implicating the medial temporal lobe system in this type of odor discrimination learning, impairments resulting from fimbria-fornix lesions in Eichenbaum et al. (1988) further imply a role for cholinergic and GABAergic septohippocampal projections in odor discrimination learning.

To replicate these findings and explicitly test the necessity of septohippocampal projections for performance in our odor discrimination learning task, we conducted preliminary experiments in which young rats received either a fornix transection, a sham surgery, or remained unsurgered as an unoperated control. Based on the results of Eichenbaum et al. (1988), in which fornix lesions profoundly impaired simultaneous odor discrimination abilities, we expected that fornix lesioned rats would be impaired in our simultaneous two-choice odor discrimination task and in the spatial version of the Morris water maze task. Unfortunately, technical and animal housing problems resulted in almost no useable data from this experiment (although there was a trend toward the fornix lesions impairing performance in the water maze). The number of fornix lesions in the preliminary study was very low, partly due to a high attrition rate associated with the medial coordinates for the lesion.

The superior sagittal sinus is anatomically located on the top of the brain, down the midline, with vasculature branching out from the sinus. Thus, physical variations in animals resulted at times in the lesion causing damage to the vasculature and extensive bleeding. Further investigations of odor discrimination or spatial learning abilities associated with the fornix may best be completed utilizing different lesion techniques to target this region while lowering the attrition rate. Possible alternative lesion strategies include an angled insertion of the electrode from more lateral coordinates, which may bypass the sinus and its vasculature. Additionally, using a knife cut instead of an electrolytic lesion may help to produce more consistent damage to the fornix, since some rats in the preliminary group could not be included due to incomplete lesions (for example Liu et al., 2002; Almaguer-Melian et al., 2006). I am currently consulting with Dr. Bizon on replicating these experiments in her new lab at University of Florida.

Age-related changes in the GABAergic system are implicated in both aged-related impairment in the water maze (Chapter II) and odor discrimination abilities (Chapter III), but it is important to acknowledge and consider that cognitive decline in the two tasks may not result solely from dysfunction in the septohippocampal pathway. Even though fornix lesions were reported to impair odor discriminations in a simultaneous two-choice odor discrimination task similar to that used in Chapter III (Eichenbaum et al., 1988), other data supports a related but distinct neural pathway in odor learning, particularly the MOB-

piriform cortex- EC pathway. Lesions of EC have been reported to impair odor discriminations that are presented simultaneously when the task requires encoding of relations between odors (as in Chapter III), but not when only memory for a single odor is necessary (Bunsey and Eichenbaum, 1993; Otto and Garruto, 1997; Wirth et al., 1998). Furthermore, disruption of olfactory ACh increases generalization between molecularly similar odors and impairs odor perceptual learning (Linster et al., 2001; Fletcher and Wilson, 2002; Wilson et al., 2004). Thus, MOB and piriform cortex may be necessary for single odor memory, whereas the EC and hippocampus and their projections from olfactory system may be involved in tasks that require multiple pieces of information pertaining to odors to be processed simultaneously. It is also possible that the septohippocampal projection may facilitate, but are not necessary for odor discrimination learning.

Even if fornix lesions do not produce deficits in odor discrimination abilities, the BF may still modulate odor discrimination learning. The relationship between spatial water maze performance and odor discrimination abilities, and the common impairment in aged animals, implicate decline in neural circuitry related to both tasks. Moreover, in same study population of F344 rats, there was an age-related increase in BF GABAergic neurons in the MS/DB, which includes projection neurons from BF to both hippocampus and MOB. Therefore, if the age-related elevation in hDB GABAergic cell number leads to an increase in GABA in the MOB, increased inhibitory signaling in MOB may result in odor

learning deficits. Moreover, systemic injections of CGP 55845 improved performance in aged-impaired rats in the simultaneous odor discrimination task; this may have been due to effects on both the olfactory and hippocampal systems whereby both odor memories (MOB/ piriform) and discriminations with multiple odors (EC-hippocampus) are enhanced. Since the BF modulates both the hippocampal and olfactory systems, it is possible that dysfunction in the BF may contribute to odor discrimination learning deficits.

Reversal of age-related impairment in olfactory learning with CGP 55845

The results of the pharmacological study presented in Chapter III utilizing CGP 55845, a GABA(B) antagonist, were in agreement with the hypothesis that an upregulation of GABAergic signaling is associated with cognitive impairment in aged rats. Young and aged rats were tested in an olfactory discrimination learning task that has been correlated with performance in the water maze. Aged rats cognitively impaired in water maze also demonstrated an increase in trials and errors to criterion on odor discrimination problems, whereas young and aged-unimpaired rats were not different in their ability to perform these odor discriminations (LaSarge et al., 2007). Using the same protocol, aged rats were classified as “aged-unimpaired” or “age-impaired” based on their ability to perform odor discrimination problems compared to young rats. Rats were then tested on the odor discrimination learning task under the influence of different doses of CGP 55845. Higher doses of CGP 55845 ameliorated odor

discrimination deficits in the age-impaired group, such that age-impaired rats performed on par with young and aged-unimpaired rats.

GABAergic bulbopetal projections from hDB influence odor memories, and indeed, lesions of the hDB produce impair olfactory memory (Wirth et al., 2000; Wilson et al., 2004; Sanchez-Andrade et al., 2005). Infusion of a GABA agonist into MOB prevents odor learning in an avoidance task (Okutani et al., 1999). If the effects of CGP 55845 are indeed specific to aged- impaired rats (see discussion under previous section), it could suggest an age-related change in sensitivity to the GABA(B) antagonist. Indeed, benzodiazepines that act on the GABA(A) system show increased efficacy of the inhibitory response that has been related to an age-related increase in the α1 subunit of GABA(A) receptors (Vela et al., 2003; Yu et al., 2006). As CGP 55845 injections in the current study were systemic, the effects of the drug were not localized to any one brain area. The enhanced performance observed selectively among aged-impaired rats therefore could be related to actions across multiple brain systems. For example, the drug could act on both the septohippocampal and olfactory-specific circuitry (although notably, CGP 55845 did not appear to enhance rats' ability to detect odors). Future experiments using intracranial infusions of GABA agonists and antagonists into specific brain regions (BF, hippocampus, and main olfactory bulb (MOB)) of young and aged rats could help delineate the specific critical circuitry on which the GABA(B) antagonist is acting to reverse learning deficits in aged-impaired rats.

Conclusion

Age-related cognitive decline occurs in the F344 rats in both spatial learning and olfactory discrimination learning. However, it has been established that natural variability exists in aged F344 rats, such that a subpopulation of aged rats maintain performance in the water maze on par with a young cohort while others demonstrate impairment. We show that aged rats impaired in the water maze had significantly increased numbers of GABAergic BF projection neurons compared to aged-unimpaired and young rats. Furthermore, among aged rats, GABAergic BF cell number was correlated with water maze performance, with higher cell numbers associated with worse performance. Cholinergic cell numbers did not differ between young and aged rats, irrespective of water maze performance. These data suggest that changes in the BF GABAergic septohippocampal system could contribute to age-related cognitive impairment.

Age-related deficits in F344 rats in a simultaneous two-choice odor discrimination task have previously been associated with impairments in the water maze. Variability in the aged population was detected, such that again some rats performed on par with young while others were impaired. However, age-related impairments were ameliorated by administration of the GABA(B) antagonist prior to performing in the task. These data suggest a role for the GABAergic system in odor discrimination task. Furthermore, combined with the age-related increase in GABAergic hDB neurons that project to MOB,

upregulation of GABA may be responsible for age-related impairment in olfactory learning.

Further experiments to link changes that occur simultaneously in the olfactory system and hippocampus, particularly in the GABAergic system, would be of great utility to the aging population. Longitudinal studies determining the specific time period in which changes in cognition and GABAergic cell upregulation in BF occur would offer insight on the inhibitory role of GABA transmission in cognitive impairment. Early detection of age-related cognitive disorders could allow for pharmacological intervention in order to halt further neuroanatomical changes that may be detrimental to cognition.

The correlative link between decline in multiple cognitive systems during aging is currently in early stages of utilization among the human population; olfactory tests such as odorant sticks have been used to detect early possible cognitive decline (Kobal et al., 1996; Eibenstein et al., 2005). However, neuroanatomical studies to determine common areas of dysfunction across these two systems would allow for a better understanding of the neurotransmitters, receptors, and other characteristics involved in cognitive impairment, possibly allowing for more treatment options. Additionally, further research into the GABAergic system may offer a valid option for combating age-related cognitive decline. Pharmacological studies are needed to determine the brain regions that the GABA(B) drug CGP 55845 is acting upon to reverse olfactory discrimination impairments, whether this type of therapeutic approach

could be extended to the human population, and if possible low dose combination therapy with other drugs (such as AChE inhibitors already in use), would offer a more efficacious approach to treating cognitive disorders while limiting side effects.

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Selected Publications:

1. LaSarge, C.L., Montgomery, K.S., Tucker, C., Slaton, G.S., Griffith, W.H., Setlow, B., & Bizon, J. L. (2007). Deficits across multiple cognitive domains in a subset of aged Fischer 344 rats. *Neurobiology of Aging* 28(6):928-36.
2. Mendez, I. A., Montgomery, K. A., LaSarge, C. L., Simon, N. W., Setlow, B. & Bizon, J. L. (2008) Long-term effects of prior cocaine exposure on spatial learning and memory. *Neurobiology of Learning and Memory* 89(2):185-91.
3. Bizon, J.L., LaSarge, C.L., Montgomery, K.S., McDermott, A.N., Setlow, B. & Griffith. W.H. (2009) Spatial reference and working memory across the lifespan of male Fischer 344 rats. *Neurobiology of Aging* 30(4): 646-55.
4. Simon, N.W., LaSarge, C.L., Montgomery, K.S., Williams, M.T., Mendez, I.A., Setlow, B, & Bizon, J.L. (in press). Good things come to those who wait: Attenuated discounting of delayed rewards in aged Fischer 344 rats. *Neurobiology of Aging*
5. Murchison, D., McDermott, A. N., LaSarge, C. L., Peebles, K. A., Bizon, J. L., & Griffith, W. H. (2009) Enhanced Calcium Buffering in F344 Rat Cholinergic Basal Forebrain Neurons is Associated with Age-related Cognitive Impairment. *Journal of Neurophysiology* 102(4): 2194-207.
6. LaSarge C., Bañuelos, C., Mayse, J. D., & Bizon J. (2009) Blockade of GABA(B) receptors completely reverses age-related learning impairment. *Neuroscience* 164(3): 941-7.

Invited Book Chapter:

LaSarge, C.L. and Nicolle, M. (2008) “Comparison of different cognitive rat models of human aging.” In Animal Models of Human Cognitive Aging. J.L Bizon and A. G. Woods, Eds. Humana Press., Totowa, NJ.