THE ROLE AND INTERACTION OF THE AT_4 AND CHOLINERGIC SYSTEMS IN THE NUCLEUS BASALIS OF MEYNERT (NBM): EFFECTS ON SPATIAL LEARNING

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of WENDY L WILSON find it satisfactory and recommend that it be accepted.

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THE ROLE AND INTERACTION OF THE AT_4 AND CHOLINERGIC SYSTEMS IN THE NUCLEUS BASALIS OF MEYNERT (NBM): EFFECTS ON SPATIAL LEARNING

Abstract

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These experiments were designed to reveal clues regarding the relationship between the AT_4 and cholinergic systems in cognitive processing. Degeneration of the cholinergic system has been hypothesized to play a significant role in Alzheimer's disease (AD) and other dementias. The NBM is a major cholinergic area, containing a dense number of cholinergic cell bodies in addition to sending widespread projections to the neocortex. It is apparent that other neural systems are involved in the devastation of this disorder. Thus, further understanding of the neural relationships underlying normal and abnormal cognitive processing is imperative for the development of therapeutics to treat cognitive ailments. Therefore, the omnibus objectives of these studies were designed to investigate the following: 1) Is the AT_4 system present in the NBM, and if so, does it play a role in cognitive processing? 2) What is the role of the

cholinergic system in the NBM, and do these systems interact or are they autonomous systems? Findings revealed that: 1) blockade of muscarinic, nicotinic or AT_4 receptors in the NBM produced significant impairments in the acquisition of the water maze task; 2) activation of the nicotinic system in the NBM reversed the AT_4-antagonist induced effects; 3) activation of the muscarinic system with carbachol did not overcome AT_4 antagonist-induced defecits; and 4) The AT_4 agonist, Nle^1-AngIV was capable of overcoming water maze acquisition deficits produced by cholinergic blockade in the NBM. Based on the collective findings of these experiments it is evident that 1) the AT_4 system does play a functional role in cognitive processing via the NBM, probably a modulatory role of the cholinergic system, and 2) a complex relationship exists between the cholinergic and AT_4 systems in this area. Possible explanations for the specific interaction between these two systems are discussed.

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To *Julie Krivsky*, without you, this would not have been possible. Thank you.

CHAPTER ONE

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Understanding normal learning and memory is not only important for basic knowledge but also to better characterize abnormalities seen in cognitive pathophysiologies such as dementia. Alzheimer's disease (AD) is the most common form of dementia. Statistics report that approximately 4.5 million individuals are currently diagnosed with AD in the U.S., and to intensify this huge number, experts predict this to triple in the next forty years (Hebert, Beckett et al. 2001; Hebert, Scherr et al. 2003). A report commissioned by the Alzheimer's Association estimated AD related costs to American businesses to approximate $61 billion a year. This includes $24.6 billion directly associated with AD health care, and $36.5 billion to businesses that employ workers who are caregivers of individuals diagnosed with AD. Estimated total cost to the nation has been speculated to be between $67 and $130 billion (Koppel 2002). The statistics above are based on the current number of 4.5 million diagnosed with AD, thus, future estimated costs to American businesses are a staggering $180 to $390 billion in the next 40 years.

AD is a severe disorder characterized by progressive and extensive decline of cognitive functions. Individuals experience mild to moderate loss of memory and information processing, followed by increasingly more severe impairments in memory, learning, reasoning, and judgment (McKhann, Drachman et al. 1984). Various neurotransmitter systems are proposed to be involved in the devastation of this disease (Gsell, Jungkunz et al. 2004). By investigating the role and interaction of these systems, a better understanding of the disease can be obtained in

addition to the possible development of novel therapeutics to attenuate or overcome the cognitive impairments of AD.

1.1 Ang IV/AT_4 System and Cognitive Processing

Angiotensin (Ang) IV is a hexapeptide resulting from the enzymatic degradation of the octapeptide Ang II. This sequence of events involves aminopeptidase action on the N-terminal amino acid residues -Asp of Ang II, and -Arg of AngIII, subsequently forming AngIV (Val-Tyr-Ile-His-Pro-Phe) (Wright, Krebs et al. 1995). Our laboratory discovered the function of this peptide in the early 1990's through the serendipitous finding of a novel angiotensin receptor subtype, now termed the AT_4 receptor (Harding, Cook et al. 1992; Swanson, Hanesworth et al. 1992). The first indication of the AngIV/AT_4 functional role came from autoradiography studies in rat and guinea pig which showed binding in brain structures linked to cognition, particularly the hippocampus, neocortex, and basal forebrain areas (Harding, Cook et al. 1992; Jarvis, Gessner et al. 1992; Swanson, Hanesworth et al. 1992; Roberts, Krebs et al. 1995). To further corroborate this distribution of receptors, intracerebroventricular (icv) injection of Ang IV was found to produce Fos-immunoreactivity (Fos-IR) in the hippocampus. Pre-treatment with other angiotensin receptor inhibitors such as losartan or PD123177 (AT_1 and AT_2 receptor antagonists, respectively), had no effect on the Ang IV-induced Fos activity, while divalinal-AngIV, (an AT_4 receptor antagonist) blocked all Fos-IR expression (Roberts, Krebs et al. 1995). Furthermore, binding studies have been performed proving similar receptor distribution patterns in both

the macaca monkey and human (Moeller, Paxinos et al. 1996; Chai, Bastias et al. 2000), indicating that AT_4 receptor location is consistent across species.

This anatomical distribution of AT_4 receptors directed the investigation of Ang IV/AT_4's functional role in learning and memory. These studies have established this system's importance in cognitive processing. For example, central administration of Ang IV analogs can either facilitate or impair learning, depending on their activity at the AT_4 receptor. Native Ang IV (Braszko, Kupryszewski et al. 1988), and analog agonists such as Nle^1-Ang IV, have proved beneficial in facilitating learning; while Ang IV antagonists such as Divalinal-Ang IV and Nle^1-$Leual^3$-Ang IV impair learning (Braszko, Kupryszewski et al. 1988; Wright, Clemens et al. 1996; Wright, Stubley et al. 1999; Pederson, Krishnan et al. 2001; Albiston, Pederson et al. 2004; Lee, Albiston et al. 2004; Olson, Olson et al. 2004). Moreover, hemorphins have been identified as endogenous peptides that can affect cognitive processing via activity at central AT_4 receptors (Moeller, Lew et al. 1997; Moeller, Chai et al. 1998). Altered levels of these peptides are seen in quantification studies of post-mortem AD brains (Poljak, McLean et al. 2004), further indicating the importance of the AT_4 receptor system in cognitive processing and associated disorders such as dementias.

1.2 The Cholinergic System and Cognitive Processing

Throughout the study of neurotransmitter systems that mediate cognitive processing, the cholinergic system has received the most attention (Rasool,

Svendsen et al. 1986; Smith 1988; Muir 1997; Mesulam 1998; Erickson and Barnes 2003). Considerable research supports the involvement of this system in both normal cognitive processing and age-related dementias. The cholinergic hypothesis of AD is derived from studies concerning the pivotal role of cholinergic ligands and receptors in learning and memory processing, as well as the observation of cholinergic degeneration in brains of patients diagnosed with AD. Pharmacological blockade of the cholinergic system is a frequently utilized pharmacological manipulation to create an animal model for studying learning and memory impairments and testing nootropic compounds. The most common drug used is scopolamine, a muscarinic receptor antagonist, which produces severe deficits in various learning and memory paradigms and is most often chosen as the pharmacological model for dementias and other amnesic disorders (Flood and Cherkin 1986; Dickson and Vanderwolf 1990; Patel and Tariot 1991)

1.3 Nucleus Basalis Magnocellularis (NBM) and Cognitive Processing

The Nucleus Basalis Magnocellularis (NBM; Meynert in primates) is an area in the basal forebrain (substantia innominata) that has received attention for its role in normal and abnormal cognitive processing (e.g. AD). The NBM is a major source of afferent cholinergic projections, particularly to the cerebral cortex and to a lesser degree to the hippocampus (Rasool, Svendsen et al. 1986; Samuel 1998). Studies investigating NBM neural cell types show this region to be densely populated with cholinergic neurons intermixed with non-cholinergic

phenotypes (Semba 2000). The cognitive impairments seen in AD and other age-related dementias are often attributed to cholinergic neurodegeneration of this area (Pepeu and Marconcini Pepeu 1994). Post-mortem studies of AD brains repeatedly show severe destruction of the NBM (Decker 1987; Pepeu and Marconcini Pepeu 1994; Teipel, Flatz et al. 2005). As expected, experimental lesions of the NBM produce profound learning and memory difficulties in various behavioral paradigms, simulating the behavioral characteristics seen in AD patients, and further supporting this structure's importance in cognition (Li, An et al. 1998; Ridley, Pugh et al. 1999; Nieto-Escamez, Sanchez-Santed et al. 2002; Nieto-Escamez, Sanchez-Santed et al. 2004).

As previously mentioned, many cognitive abnormalities have been attributed to cholinergic disruption. However, a number of studies have shown that highly selective cholinergic neurotoxins do not completely impair learning (Page, Everitt et al. 1991; Baxter, Bucci et al. 1995). Thus, experiments have been done to test different neurotoxic agents directly infused into the NBM, the amount of resulting cholinergic cell loss, and its correlation with impaired performance in learning-associated tasks. For example, immunotoxic lesions of the NBM with either ibotenate or quisqualate found that both were equally specific to choline acetyltransferase (ChAT) immunoreactive cells and produced similar decreases of cortical ChAT positive cells. However, only ibotenate produced deficits in T-maze performance (Wenk, Harrington et al. 1992). These results 1) questioned the importance of the cholinergic system in cognition, and 2) pose the idea that an additional neurotransmitter system(s) is important in learning. Cell destruction of

this system is likely producing such behavioral impairments. In a similar study, alpha-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA) or ibotenic acid were assessed for their ability to deplete cortical cholinergic cells and the resulting impact of their depletion on performance in the water maze task. AMPA lesions decreased cortical ChAT levels by 70% but had no effect on learning, while ibotenic acid reduced ChAT levels by only 50% and significantly impaired water maze acquisition (Page, Everitt et al. 1991), again indicating a lack of association between cholinergic loss and behavioral impairment. Furthermore, 192 IgG-saporin (a highly selective cholinergic neurotoxin) infused into the NBM did not impair animals in the acquisition phase of the Morris water maze task (Baxter, Bucci et al. 1995). Taken together these findings implicate the additional importance of non-cholinergic cells in cognitive processing mediated by the NBM. Furthermore, it remains questionable whether the AT_4 system is autonomous or is in fact acting through the cholinergic system (see fig 1). Thus, investigating the potential role of other neurotransmitter systems and their interaction with the cholinergic system in the NBM is imperative for several reasons: 1) to further our knowledge of the NBM's role in cognition; 2) to determine whether AT_4 effects are cholinergic dependent, and 3) to facilitate the development of novel non-cholinergic drug therapies for the treatment of dementias such as AD.

To attempt to address the aforementioned goals, a series of studies were conducted using pharmacological tools to explore interactions between the AT_4 and cholinergic systems (see Table 1 for drugs used and/or mentioned in these experiments). The goals of the first paper, *The role of the AT_4 and cholinergic*

systems in the Nucleus Basalis Magnocellularis (NBM): Effects on Spatial Memory, were: 1)to determine if AT_4 receptor antagonists infused into the NBM impair acquisition of the water maze task (providing evidence for a functional role of this system in the NBM); 2) to determine if intra-NBM cholinergic antagonists interfere with water maze acquisition; and 3) to ascertain if muscarinic and nicotinic cholinergic agonists can overcome AT_4-induced deficits in the circular water maze.

The goal of the second paper, *The role of Nle^1-AngIV in Spatial Memory in Nucleus Basalis Magnocellularis (NBM) Scopolamine-Induced Deficits in Rats,* were designed to further evaluate the interaction of the AT_4 and cholinergic system in the NBM. Specifically, this study was designed to assess the capability of Nle^{1}AngIV to overcome NBM-cholinergic blockade induced effects in the water maze task.

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Table 1

Agonist versus Antagonist activity of cholinergic and AT_4 receptor ligands

Compound	Receptor System	Activity
ACh	mAChR and nAChR	Agonist
AngIV	AT_4 Receptor	Agonist
Carbachol	mAChR (M_2)	Agonist
Divalinal-AngIV	AT_4 Receptor	Antagonist
Gallamine	mAChR (M_2)	Antagonist
Hemorphins (LVV-Hemorphin-7)	AT_4 Receptor	Agonist
Mecamylamine	nAChR	Antagonist
Nle^1AngIV	AT_4 Receptor	Agonist
Nle^1Neul^3AngIV	AT_4 Receptor	Antagonist
Nicotine	nAChR	Agonist
Oxotremorine	mAChR	Agonist
Pilocarpine	mAChR (M_1)	Agonist
Pirenzepine	mAChR (M_1)	Antagonist
Scopolamine	mAChR	Antagonist

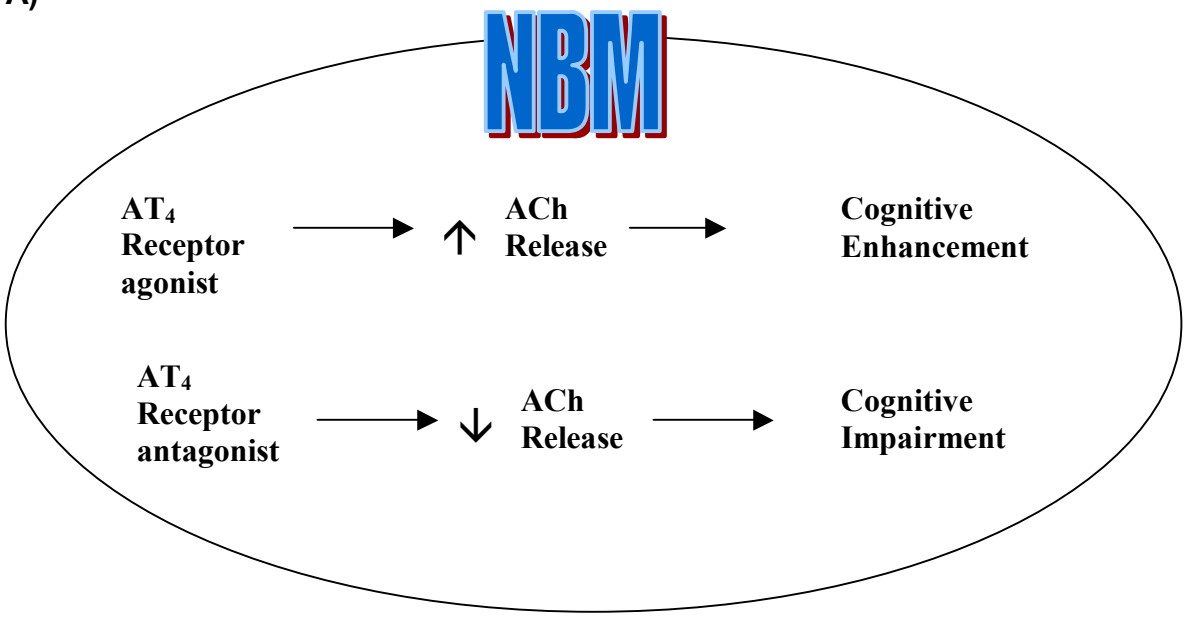
Acetylcholine (ACh), Angiotensin IV (AngIV), muscarinic Acetylcholine Receptor (mAChR), mAChR subtypes (M_1-M_5), nicotinic Acetlycholine Receptor (nAChR).

Fig 1. Possible relationships between the AT_4 system and cholinergic system on cognition in the NBM

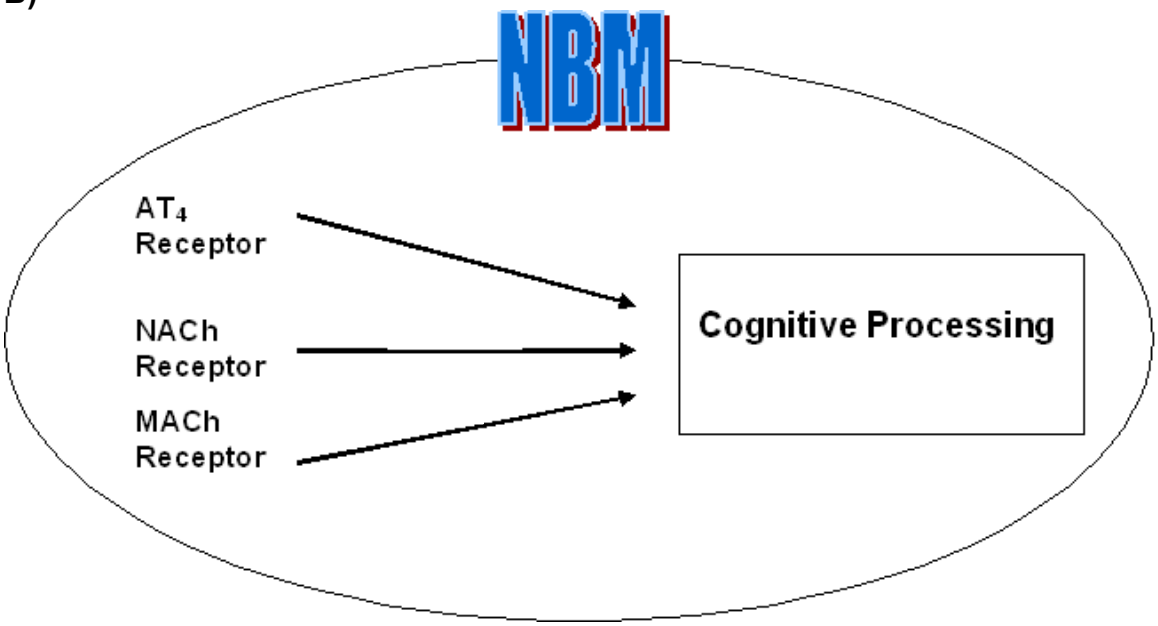
A) AT_4 receptor system mediates cognitive function through regulation of cholinergic transmission. AT_4 receptor activation stimulates cholinergic release which acts on cholinergic receptors to facilitate cognition. Correspondingly, AT_4 receptor inhibition decreases cholinergic transmission resulting in decreased cholinergic receptor activation and cognitive impairment.

B) The AT_4 and cholinergic receptor systems are autonomous. The integrity of all three receptor systems (AT_4, muscarinic, and nicotinic) are required for normal cognitive processing. Blocking any one of the receptor systems produces cognitive deficiencies. However, this blockade can be overcome by increasing activity of one of the non-impaired receptor systems resulting in normal cognitive processing.

A)


NBM
AT4 Receptor agonist
ACh Release
Cognitive Enhancement
AT4 Receptor antagonist
ACh Release
Cognitive Impairment

B)


NBM
AT4 Receptor
NACh Receptor
MACh Receptor
Cognitive Processing

CHAPTER TWO

THE ROLE OF THE AT_4 AND CHOLINERGIC SYSTEMS IN THE NUCLEUS BASALIS MAGNOCELLULARIS (NBM): EFFECTS ON SPATIAL MEMORY

THE ROLE OF THE AT_4 AND CHOLINERGIC SYSTEMS IN THE NUCLEUS BASALIS MAGNOCELLULARIS (NBM): EFFECTS ON SPATIAL MEMORY

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Abstract

The brain AT_4 and cholinergic systems play a pivotal role in learning and memory. Many studies have investigated the nootropic and amnesic properties of both systems. The cholinergic system has received the most attention and appears to contribute to normal and abnormal cognitive functioning. For example, one of the best known cognitive disorders, Alzheimer's disease (AD), is treated with cholinergic-directed drugs, and post-mortem studies of AD patient brains show neurodegenerative devastation in cholinergic areas of the brain. Recent studies have suggested that potentiation of cholinergic transmission may be a mechanism by which the AngIV/AT_4 receptor system enhances cognition (Lee, Chai et al. 2001; Olson, Olson et al. 2004). Since the Nucleus Basalis Magnocellularis/Meynert (NBM) (in primates) is a main source of cholinergic innervation to major cognitive areas of the brain, this site was chosen to investigate the role and interaction of the two systems. Sprague-Dawley rats were fitted with permanent bilateral cannulas targeting the NBM through which all compounds were bilaterally administered. Pre-treatment with divalinal-AngIV, an AT_4 receptor antagonist produced profound deficits in performance in the circular water maze. Pretreatment with nicotine completely reversed these divalinal-AngIV induced impairments. In contrast, carbachol, a muscarinic receptor agonist, did not attenuate this impaired acquisition, and at higher doses appeared to exaggerate the divalinal induced-deficits. Similar to the AT_4 antagonist, both scopolamine and mecamylamine (muscarinic and nicotinic receptor antagonists, respectively), prevented acquisition of the water maze task.

Based on these results, it appears that blocking any one of these systems results in impaired spatial learning, while activating the nicotinic receptor system counteracts the effects of AT_4 receptor blockade. These findings suggest a functional role for both the cholinergic and AT_4 receptor systems in spatial learning, and indicate for the first time a functional role for the AngIV/AT_4 receptor system in the NBM.

Abbreviations

ACh, acetylcholine; aCSF, artificial cerebrospinal fluid; AngIV, angiotensin IV; ICV, intracerebroventricular; NBM, Nucleus Basalis Magnocellularis (Rat)/Meynert (human)

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by severe memory and learning impairments. Typical features of AD include a rapid deterioration of memory loss in conjunction with the inability to consolidate new information. This eventually progresses to widespread multi-neuronal system loss, with multiple accompanying behavioral pathologies. Currently, AD can only be positively diagnosed through post-mortem analysis, i.e. presence of amyloid plaques, neurofibrillary tangles, and degeneration of cholinergic neuron populations. This loss of cholinergic neurons is referred to as the "cholinergic hypothesis", which is believed to be the primary cause of the cognitive symptoms characteristic of AD. A considerable amount of evidence supports the role of the cholinergic system in cognition. For example, cholinergic blockade with antagonists produces severe memory impairments in many animal and human learning paradigms, whereas cholinergic agonists can often overcome memory impairments produced through lesions or pharmacological disruption.

Anatomical distribution of brain AT_4 receptors is very closely linked to the localization of brain cholinergic receptors, particularly in areas under scrutiny for their role in learning and memory. A number of studies have explored these two systems with respect to their dual or counter-effects on cognitive processing. For instance, administration of scopolamine (a muscarinic receptor antagonist) produces severe deficits in various learning or memory tests, and is most often chosen as the pharmacological model for dementias and other amnesic disorders (Flood and Cherkin 1986; Dickson and Vanderwolf 1990; Patel and

Tariot 1991). To assess the possibility of overcoming scopolamine-induced deficits, our laboratory as well as others, have investigated the role of various AT_4 ligands in scopolamine-treated animals. Nle^1-AngIV (AT_4 receptor agonist) can overcome scopolamine-induced deficits in the acquisition phase of the Morris water maze task, while co-administration of Nle^1Leual^3AngIV (an AT_4 receptor antagonist) blocked this Nle^1-AngIV-induced compensatory effect. These findings suggest that cognitive enhancing properties of AT_4 are mediated through AT_4 receptor activity (Pederson, Harding et al. 1998; Pederson, Krishnan et al. 2001). Studies utilizing AT_4 agonists such as Nle^1AngIV, LVV-hemorphin and native AngIV have replicated these findings in passive avoidance and water maze studies (Braszko, Kupryszewski et al. 1988; Albiston, Pederson et al. 2004; Lee, Albiston et al. 2004). Recently, Olson et al. (2004) tested the ability of Nle^1AngIV to overcome mecamylamine (a nicotinic receptor antagonist) or scopolamine-induced impairments. As expected, Nle^1-AngIV could overcome mecamylamine- or scopolamine-induced deficits; however, this facilitatory activity could not overcome the impairments produced by the combination of these two drugs, i.e. a scopolamine/mecamylamine cocktail (Olson, Olson et al. 2004). One potential explanation for these results is that the cognitive effects of the AT_4 system occur via altering cholinergic activity. To test this hypothesis, Lee et al. (2001) treated hippocampal slices with Ang IV or LVV-hemorphin-7 and tested their effects on cholinergic transmission. Both AT_4 agonists provoked cholinergic release from the hippocampus in a dose-dependent fashion. To further determine if this cholinergic release was AT_4 receptor-mediated, slices were

pretreated with divalinal-AngIV (an AT_4 antagonist) in the presence of Nle^1-AngIV. Divalinal-AngIV blocked the ability of Nle^1-AngIV to provoke acetylcholine release from the hippocampal slices, indicating the importance and specificity of the AT_4 receptor in this process (Lee, Chai et al. 2001).

The objective of the present study was to assess the interaction between the cholinergic system and the AT_4 system, and ultimately their functional and mechanistic roles in cognitive processing, specifically spatial learning. Autoradiographical studies have found a high propensity of these receptor systems in areas known to be involved in learning and memory processing. The present study focused on the NBM because: 1) it is a major cholinergic projection area; 2) it appears to be significantly involved in the pathology of AD; 3) the integrity of this area is important in cognitive function; and 4) the AT_4 system's functional role in the NBM has not previously been investigated. The NBM has been shown to exhibit significant neurodegeneration in post-mortem studies of Alzheimer's patients. By further investigating the role and interactions of these two systems a better understanding of the disease can be obtained in addition to providing insight concerning novel treatments to attenuate the cognitive impairments seen in AD. This study was designed to answer the following question: what is the role and interaction of the AT_4 and cholinergic systems in the NBM on spatial learning? Specifically, can direct infusions of cholinergic or AT_4 compounds alter acquisition of the water maze task, and if so can boosting one system overcome blockade-induced deficits of the other?

2. Methods

Animals and surgery

Male Sprague-Dawley rats weighing 350-550 g were housed in an AAALAC-approved vivarium maintained at 22 ± 1 °C, with a 12:12 h light cycle initiated at 07:00 h. All animals were allowed ad libitum access to rat chow (Harland Teklad Rodent Diet, Madison, WI) and tap water throughout the experiment. The rats were anesthetized with Equi-thesin (0.3 ml/100 g ip), and lidocaine (Phoenix Pharmaceutical, Inc. St.Joseph, MO) was administered as a local anesthetic. Topical betadine (Fabrique Par H&P Industries Inc. Mukwonago, WI) was applied following surgery to prevent post surgical infection. All rats were fitted with a chronic bilateral cerebral NBM guide cannula (PE-60, Clay Adams. Sparks, MD, length = 2.5 cm) by sterotaxic surgery positioned above the NBM. Coordinates were 1.3 mm posterior to bregma and 3.0 mm lateral from the midline. A heat bulge, positioned 2.5 mm from the tip of the cannula, controlled the depth of penetration below the skull. The cannula was secured with stainless steel screws and dental cement after stereotaxic placement. Four days following surgery, animals were handled for approximately 5 min per day for 3 days. Following behavioral testing correct cannula placement was verified postmortem with an injection of green dye infusion into the cannulas followed by histology.

Compounds

Divalinal-AngIV (Pacific Northwest Biotechnology, Pullman, WA), scopolamine (Sigma-Aldrich Co., St. Louis, MO), mecamylamine (Sigma-Aldrich,

Co. St. Louis, MO), nicotine (Sigma-Aldrich Co., St. Louis, MO), and carbachol (Sigma-Aldrich Co., St. Louis, MO) were each dissolved in aCSF.

Divalinal-AngIV was administered at a dose of 10 nmol/μl aCSF (1μl total volume) and scopolamine at a dose of 17.5 nmol/μl, bilaterally into the NBM.

Nicotine was administered at a dose of 1μg/μl aCSF, mecamylamine 0.3 ug/μl, and carbachol at 4 different doses: 1 μg/μl, 0.5 μg/μl, 0.25 μg/μl, and 0.10 μg/μl aCSF; all were administered bilaterally into the NBM in a volume of 1 μl/injection. These doses were previously found to have a facilitatory effect on acquisition training in other learning paradigms (Barros, Ramirez, Reis, Izquirdo, 2004).

All infusions were administered via 10 μl Hamilton microsyringes, attached to PE 20 tubing, hand-delivered over a period of 30 s. The injector consisted of 29-gauge stainless steel tubing, extending 4.3 mm beyond the length of the guide cannula (2.5 cm).

Circular water maze

The water maze apparatus was a circular black galvanized tank (diameter: 1.6 m; height 0.6 m), filled with water kept at a temperature of 26-28 °C. The maze was operationally partitioned into four equal quadrants of NW, NE, SW and SE. Animals began each trial at a different entry point, facing the wall of the tank. The entry points were denoted as one of the four quadrant corners (i.e. N, S, E and W) and were randomly assigned per trial. Extra-maze spatial cues

consisted of different colored cardboard shapes (circles, squares and triangles) on three of the four walls in the testing room.

Acquisition trials were run on eight consecutive days, with five trials conducted per day. The trials required the rat to locate a submerged pedestal (2 cm below the water line), and was placed in one of the four quadrants and remained fixed for the duration of acquisition training. The rat was allowed 120 s per trial to locate the pedestal. Once the animal found the pedestal, it was allowed a 30 s rest period. If the rat failed to locate the pedestal, the experimenter placed the animal on the pedestal for the 30 s rest period. Immediately following the rest period the next trial ensued. Swimming path was analyzed by a computer video tracking system (Chromotrack, San Diego Instruments, San Diego, CA). The computer recorded total swim latency to locate the pedestal, in addition to total swim distance per trial. Swim speed was calculated by dividing the swim distance by the swim latency.

Experiment 1: Can divalinal-AngIV, scopolamine, or mecamylamine delivery into the NBM alter spatial learning?

Prior to drug administration, animals were randomly chosen to be in one of four groups (8 animals per group). The treatment groups received an injection of either divalinal (1nmol/1μl aCSF/cannula), scopolamine (35 nmol1μl aCSF/cannula), or mecamylamine (0.3 μg/1μl aCSF/cannula) 5 min prior to behavioral testing in the water maze. The control group was given the same volume, but vehicle (aCSF) only.

Experiment 2: Can nicotine overcome divalinal-induced impairments?

Animals were again randomly chosen to be in one of two groups (8 animals per group). Group one consisted of the treatment group in which animals were administered nicotine (1ug/1μl aCSF/cannula) into the NBM 15 min prior to training. Five min prior to training each animal received an injection of divalinal (1nmol/1μl aCSF/cannula). Group two received the same injection schedule, but was given only the vehicle (aCSF).

Experiment 3: Can carbachol overcome divalinal-induced impairments?

Animals were randomly chosen to be in one of three groups. In the treatment group animals were administered carbachol followed 10-15 min later with divalinal. Experiments were run using carbachol at two different doses (0.5ug/1ul (n=4), 0.25/1ul (n=3) aCSF/cannula). The carbachol was administered 15 min prior to training followed by divalinal (1nmol/1ul aCFS/cannula) 5 min prior to the water maze task. The control group received the same injection schedule, but drugs were replaced with a vehicle injection (aCSF).

Experiment 4: Does carbachol impair spatial learning?

Animals were randomly chosen to be in one of two groups (6 animals per group). In group one, animals were administered carbachol only at two different doses (0.5ug/1ul and 0.1ug/1ul aCSF/cannula). The carbachol was administered

15 minutes prior to training. The second group, the control group, received a vehicle injection (1ul aCSF/cannula) 15 minutes prior to training.

Statistics

Latency and swim speed to find the pedestal per day (mean of 5 trials each day) were analyzed using a two-way ANOVA, with groups being between subjects and days of acquisition within subjects.

The significance level was set at $P<0.05$. Post-hoc analyses were performed using LSD test ($P<0.05$) in order to evaluate significant differences. A one-way ANOVA was performed on the latencies for the last day of testing to distinguishing group differences. Data are presented as mean ± S.E.M.

3. Results

Experiment 1: Effects of Divalinal-AngIV, Scopolamine, or Mecamylamine delivery into the NBM on spatial learning

A 4 (groups) X 8 (days) ANOVA indicated a significant groups effect in latency to find the submerged platform: [$F(3,26) = 13.72$, $p<0.001$], in addition to a significant days effect: [$F(7,20) = 4.44$, $p < 0.005$], but no interaction effect: [$F(21,58) = 1.56$, $p>0.05$]. As expected, latency to find the pedestal decreased with the number of trials (5/day) performed, see Fig. 1. LSD post hoc analysis revealed a significant difference between the control group and each of the three treatment groups, divalinal-AngIV, scopolamine, and mecamylamine, $P<0.001$. No differences were seen among the three treatment groups. A one-way

ANOVA for latency to find the platform was performed on Day 8, which additionally found that rats given an antagonist were significantly slower in finding the pedestal than control rats [F (3,26)=18.5, *P < 0.001*].

To evaluate possible locomotor dysfunction or sickness/drug toxicity, swim speeds were calculated for each group. A Group (4) X Day (8) ANOVA indicated that there was no difference among groups concerning swim speed, [F(3,26) = .325, p> 0.05].

Experiment 2: Reversal Effects of nicotine on divalinal-AngIV induced impairments

A 3 (groups) X 8 (days) ANOVA found a main effect of group in latency to reach the pedestal (F(2,21)=5.52, p< 0.05) and a main effect of number of trials completed (F(7,15) =1.52,p< 0.001), (refer to Fig. 2). LSD post-hoc analysis indicated that the nicotine + divalinal-AngIV group was not significantly different from the control group, whereas the divalinal-AngIV group was different from the vehicle group (p< 0.005). A one-way ANOVA was also used to analyze the final day of testing, which indicated a difference in ability to find the pedestal across groups [F(2,23)=10.29, *P<.001*]. LSD post hoc analysis concluded that the nicotine/divalinal group was not different than controls but did differ from the divalinal only group. An additional one-way ANOVA performed on the data from the last test day (day 8) showed that latencies to find the pedestal were significantly different between divalinal and control groups, [F(2,23)=10.29, *P*<.001], however, the nicotine/divalinal group was not significantly different from controls.

Swim speed analysis (ANOVA) demonstrated no difference among groups, [F(2,21)=0.295, P>.05]; indicating that all rats swam at approximately the same speed; thus no locomotor or toxicity effects appeared to be induced by the drugs.

Experiment 3: Carbachol effects on divalinal-induced impairments

A 3 (groups) X 8 (day) ANOVA found a main effect of group [(F(2,10)=14.21, *P*<.001)] (Fig 3). Swim speed analysis indicated no difference among groups, indicating that the treatment rats did not swim slower than controls, which would be expected if drugs reached toxic levels.

Experiment 4: Is Carbachol inhibitory

Statistical analysis using a 3 (groups) X 8 (days) ANOVA found a main effect for groups [(F(2,15)=7.57, *P*<.005)] and days of training [(F(7,9)=6.35, *P<.05)*]. LSD post hoc analysis indicated that the control group was significantly different from the 0.5 μg carbachol dose (*P*<0.001) but not the 0.1 μg dose. These results suggest that the 0.1 μg dose was not consistently effective. However, analysis of the 3 groups on the last day of training, day 8, found that both the 0.1 and 0.5 μg doses were significantly different from controls [(F(2,17)=32.94, *P*<.05].

Swim speed analysis (ANOVA) found no significant differences across groups.

4. Discussion

Results of this study indicated that intra-NBM injections of the AT_4 antagonist, divalinal, impaired acquisition of the water maze task, demonstrating for the first time that the AT_4 system in the NBM has a functional role in mediating spatial learning. This impairment agrees with other studies utilizing AT_4 receptor antagonists (Nle^1-$Leual^3$-AngIV or divalinal-AngIV) which have been shown to inhibit learning in water maze and passive avoidance tasks following intracerebroventricular (icv) administration (Braszko, Kupryszewski et al. 1988; Wright, Stubley et al. 1999).

Experiment 1 also investigated the ability of the cholinergic antagonists to impair water maze performance following intra-NBM administration. As presented in Fig. 1 both scopolamine and mecamylamine were equally capable of severely compromising water maze acquisition. Based on these findings it appears that the integrity of all three systems is required for spatial-dependent learning to occur, i.e. inhibition or blockade of any one of the three systems is detrimental to acquisition of the water maze task.

To further investigate this functional network we assessed the ability of cholinergic agonists to overcome AT_4 receptor blockade-induced deficits. These studies were designed to evaluate the interactions of the two cholinergic systems with the AT_4 system, primarily focusing on the question: can increased activation of one receptor subtype overcome the behavioral deficits produced by blocking one of the others? As presented in Fig. 2, nicotine infused into the NBM was successful in overcoming divalinal-AngIV-induced impairments. This facilitation

agrees with a number of reports showing nicotine's ability to improve cognition in humans and rodents following various cognitive impairments (Newhouse, Potter et al. 2004; Buccafusco, Letchworth et al. 2005). Lee (Lee, Chai et al. 2001) found that AT_4 receptors located on the presynaptic terminal are capable of releasing ACh into the synapse when activated in hippocampal slices. This could also be the case in the NBM. Based on these findings it is conceivable that divalinal blocks pre-synaptic ACh release, while mecamylamine and scopolamine block ACh binding at the post-synaptic receptor. Each of these scenarios may ultimately impact cholinergic function resulting in compromised spatial abilities. Findings of experiment 2 suggest that nicotine can bypass presynaptic ACh release by activating nAChRs and overcoming the spatial impairments. However, one problem with this interpretation is the previous findings that divalinal had no effect on basal ACh release (Lee, Chai et al. 2001; Lee, Albiston et al. 2004). One possible explanation for these differing results is that the dose of divalinal used in these studies was sufficient to interfere with Nle^1AngIV agonist binding, but was not high enough to produce behavioral changes (i.e. a lack of sufficient receptor blockade to modify function). Another possibility is that receptor activity and/or function could be site-dependent. To support this notion, intraseptal infusions of divalinal-AngIV did not impair spatial learning in the water maze (Wilson, unpublished findings). The medial septum provides cholinergic projections to the hippocampus; in Lee's studies the divalinal was either infused directly onto slices of hippocampal tissue or infused into the lateral ventricles, whereas in this study the infusion was targeted directly into the NBM. It is

questionable whether or not infusions into the lateral ventricles are able to reach the basal forebrain, specifically the NBM. This inability of icv divalinal to diffuse to the NBM could account for the differing effects between the present and previous findings.

In contrast with the nicotine findings, carbachol, the mAChR agonist did not overcome divalinal-AngIV induced learning deficits (see Fig.3), and under closer evaluation may have even exacerbated the behavioral effects of the AT_4 antagonist. This notion was further explored by intra-NBM administration of carbachol alone. As seen in Fig. 4, carbachol at two different doses interfered with acquisition of the water maze task. Results of studies assessing the effects of the mAChR agonist carbachol on cognitive processing are mixed. While there is evidence to suggest that muscarinic agonists facilitate learning and memory (Markowska, Olton et al. 1995; De-Mello, Souza-Junior et al. 2005) the results appear to depend on the compound's affinity for the different subtypes of mAChR. For example, subcutaneous pilocarpine (M_1 agonist) or intra-septal carbachol (M_2 agonist) have been shown to overcome scopolamine-induced deficits in the 12-arm radial maze and T-maze, respectively (Dennes and Barnes 1993; Givens and Olton 1994). Arecholine, pilocarpine (M_1 agonists), or high doses of carbachol (M_2 agonist) facilitated learning in the active-avoidance task (Sen and Bhattacharya 1991); However lower doses of carbachol were found to impair learning. The authors attributed these dose-dependence differences to receptor specificity. At higher doses carbachol was believed to act on additional muscarinic receptor subtypes, while at lower doses it appeared to have a higher

specificity for the M_2 receptor subtype. To further complicate the interpretation, several investigations have suggested that behavioral activity mediated by carbachol depends on many factors including dose, amount of cholinergic neurotransmission during administration, timing of dose (i.e. before learning, after learning, etc.) and route of administration/site of microinfusion (Sen and Bhattacharya 1991; Siniscalchi, Badini et al. 1992; Bunce, Sabolek et al. 2004).

Thus, carbachol can have opposing effects on learning and memory depending on the amalgamation of the above factors. These contradictory findings with carbachol hint at the complexity of the muscarinic system in learning and memory. For example, M_1 receptor agonists have been shown to overcome age-related deficits in water maze learning (De-Mello, Souza-Junior et al. 2005), while both M_1 receptor agonists and M_2 receptor antagonists are cognitive enhancers during acquisition of the active avoidance paradigm (Sen and Bhattacharya 1991). Carbachol has been indicated to have highest affinity for the M_2 receptor (Sen and Bhattacharya 1991). A number of reports have described the M_2 receptor as an autoreceptor modulating cholinergeric transmission in the hippocampus and cholinergic basal forebrain areas (Mesulam 1998; Rouse, Edmunds et al. 2000). The M_2 receptor has been reported to be the dominant cholinergic receptor in the NBM (Mesulam 1998). To further support the importance of the M_2 autoreceptor, Siniscalchi et al., 1992 proposed that the M_2 receptor subtype provides a negative feedback mechanism which inhibits electrically stimulated ACh release in NBM slices (Siniscalchi, Badini et al. 1992). Based on this evidence it seems likely that intra-NBM administration of

carbachol activates M_2 autoreceptors reducing cholinergic release at the synapse and thus obstructing cholinergic neurotransmission, which is known to induce learning deficits.

The present results indicate that the cholinergic and AT_4 systems participate functionally in cognitive processing via the NBM. It remains unclear whether these two systems are autonomous or the AT_4 system is producing behavioral effects through cholinergic neurotransmission. However, it is evident that disruption of the neurochemistry of this small basal forebrain area negatively impacts proper cognitive processing, and the cholinergic and AT_4 systems play a vital role in this functioning. Furthermore, the severe learning deficits resulting from pharmacological manipulation of these systems suggests that the NBM may be equivalent to the hippocampus, if not more important to learning and memory. It is well established that proper functioning of the hippocampus is a necessity for normal learning and memory; for example, lesioning or interfering with hippocampal functioning results in learning impairments (Volpicelli-Daley, Duysen et al. 2003; Sweatt 2004; Wright, Murphy et al. 2004,Volpicelli-Daley, 2003). In conclusion, the cholinergic and AT_4 systems' interaction in the NBM should be explored further to fully understand the mechanisms of this area in mediating cognition, with a specific focus on the AT_4 system in therapeutic strategies to possibly enhance the meager effects of current AD drugs.

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Figure Captions

Fig. 1. Effects of intra-NBM administration of 10 nmol divalinal-AngIV (n=8), 17.5 nmol scopolamine (n=8), 0.3 μg mecamylamine (n=8), or vehicle (aCSF) (n=8) on spatial learning. The data are expressed as mean ± SEM (averaged over 5 trials per session). All antagonists were significantly different from the control group (P<.05).

Fig. 2. Effects of intra-NBM administration of 10 nmol divalinal-AngIV (n=8) or divalinal pretreated with 1μg nicotine (n=8) , versus aCSF vehicle controls (n=8) on acquisition of the water maze task. The data are expressed as mean ± SEM (averaged over 5 trials per session). There was a significant group difference in latency compared to control on day 8 (* *P*<.001).

Fig. 3. Effects of intra-NBM administration of carbachol (0.25 μg, n=3 or 0.5 μg, n=4) pretreated with divalinal-AngIV versus aCSF controls (n=6) in the water maze task. The data are expressed as mean ± SEM (averaged over 5 trials per session). All treatment groups were significantly different from the control group on day 8 (*P<.05).

Fig. 4. Effects of intra-NBM administration of carbachol alone (0.1 μg or 0.5 μg) (n=6 each group) administration versus aCSF controls (n=6) in the water maze

task. The data are expressed as mean ± SEM (averaged over 5 trials per session). Both carbachol groups differed from the control group ($P<.05$).

Figure 1

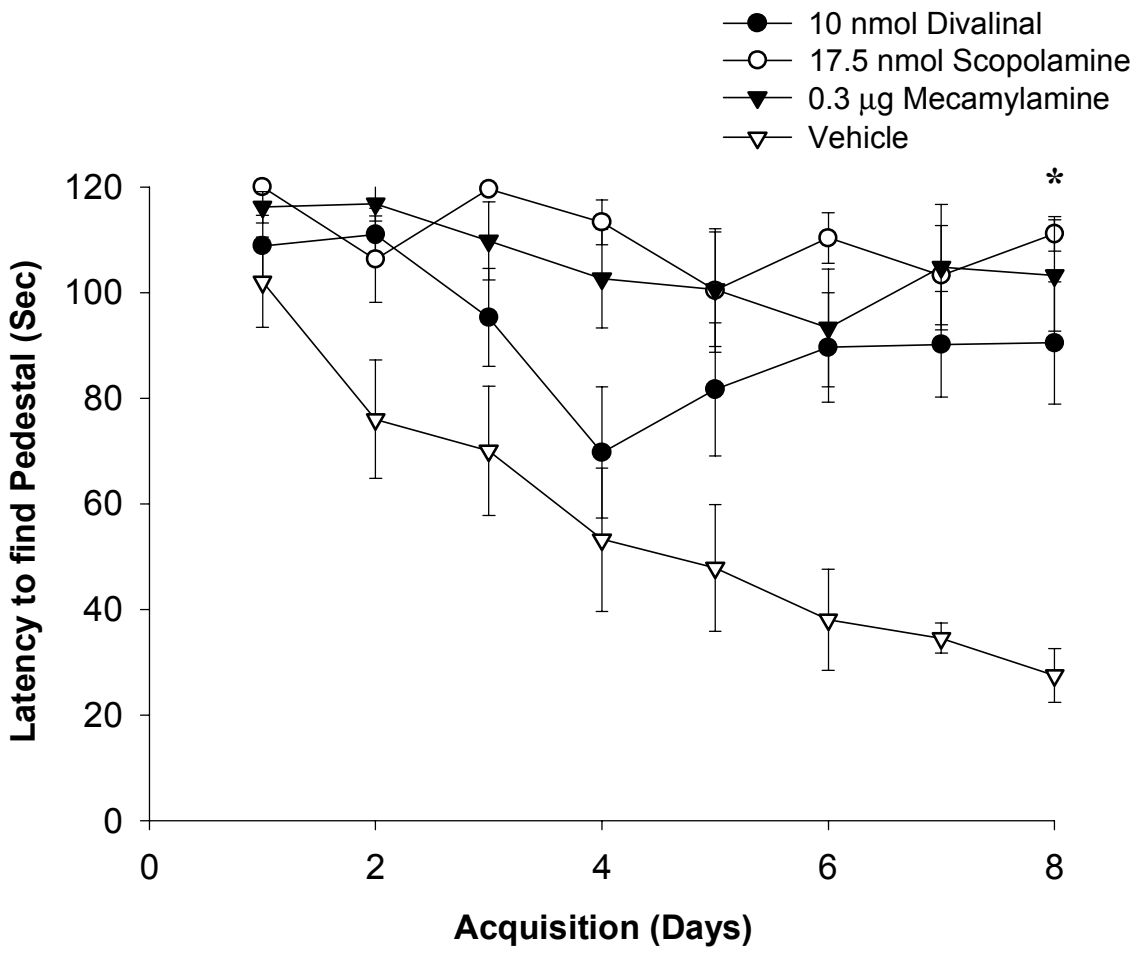

10 nmol Divalinal
17.5 nmol Scopolamine
0.3 μg Mecamylamine
Vehicle
*
Latency to find Pedestal (Sec)
120
100
80
60
40
20
0
0
2
4
6
8
Acquisition (Days)

Figure 2

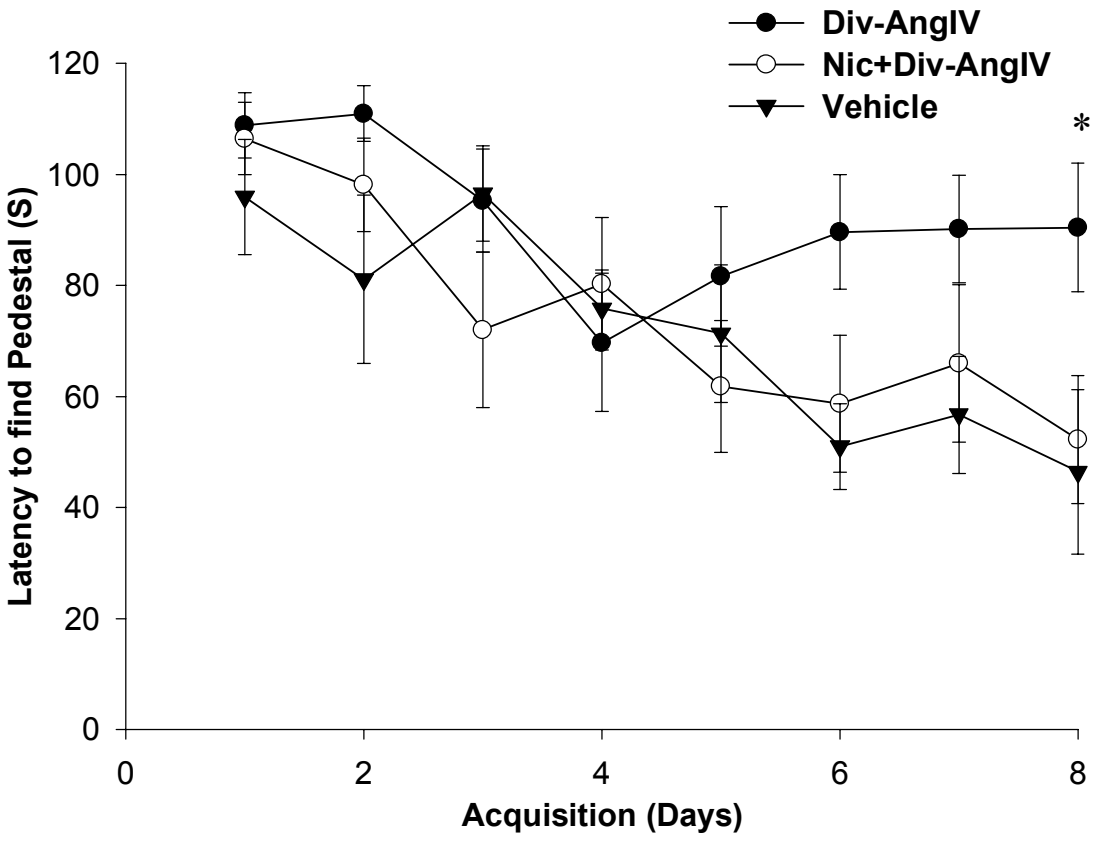

Div-AnglV
Nic+Div-AnglV
Vehicle
*
Latency to find Pedestal (S)
120
100
80
60
40
20
0
0
2
4
6
8
Acquisition (Days)

Figure 3

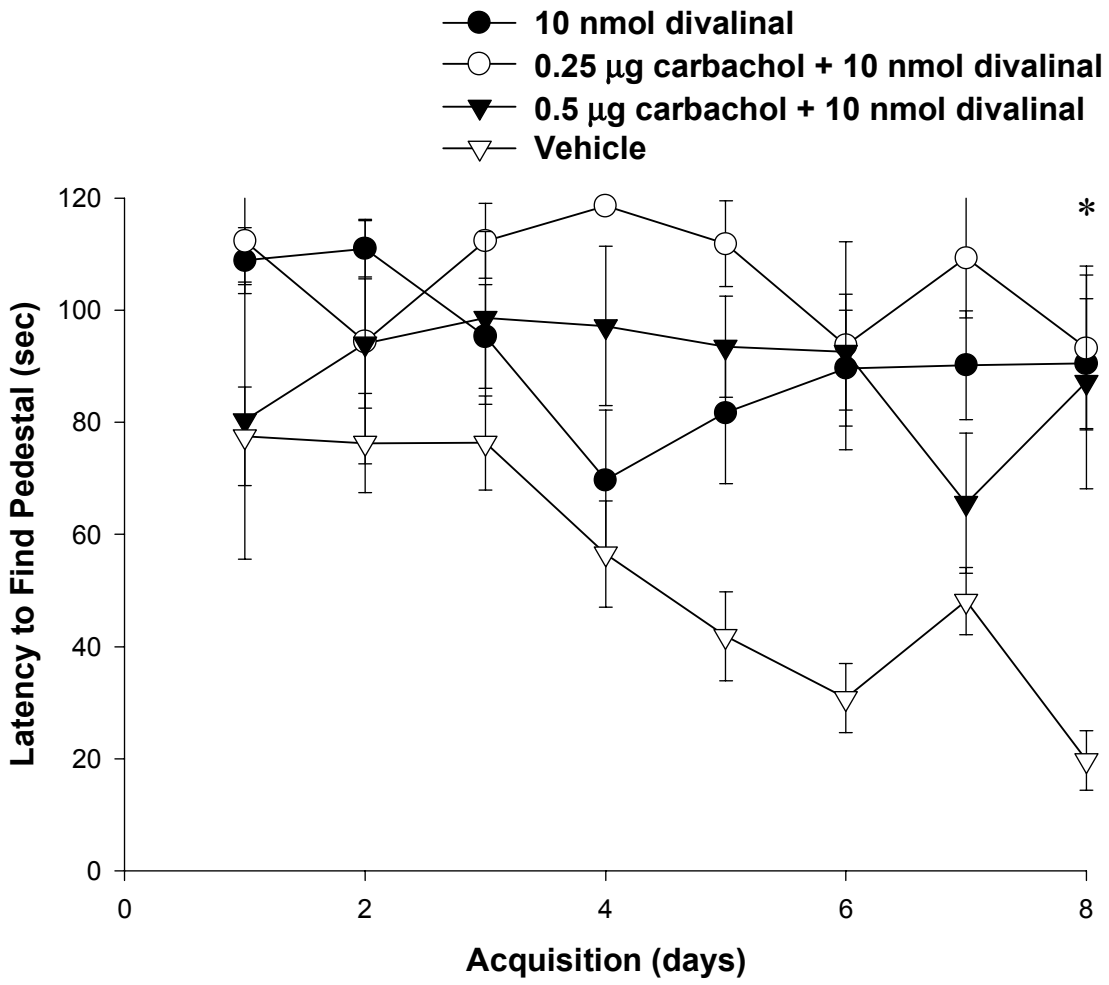
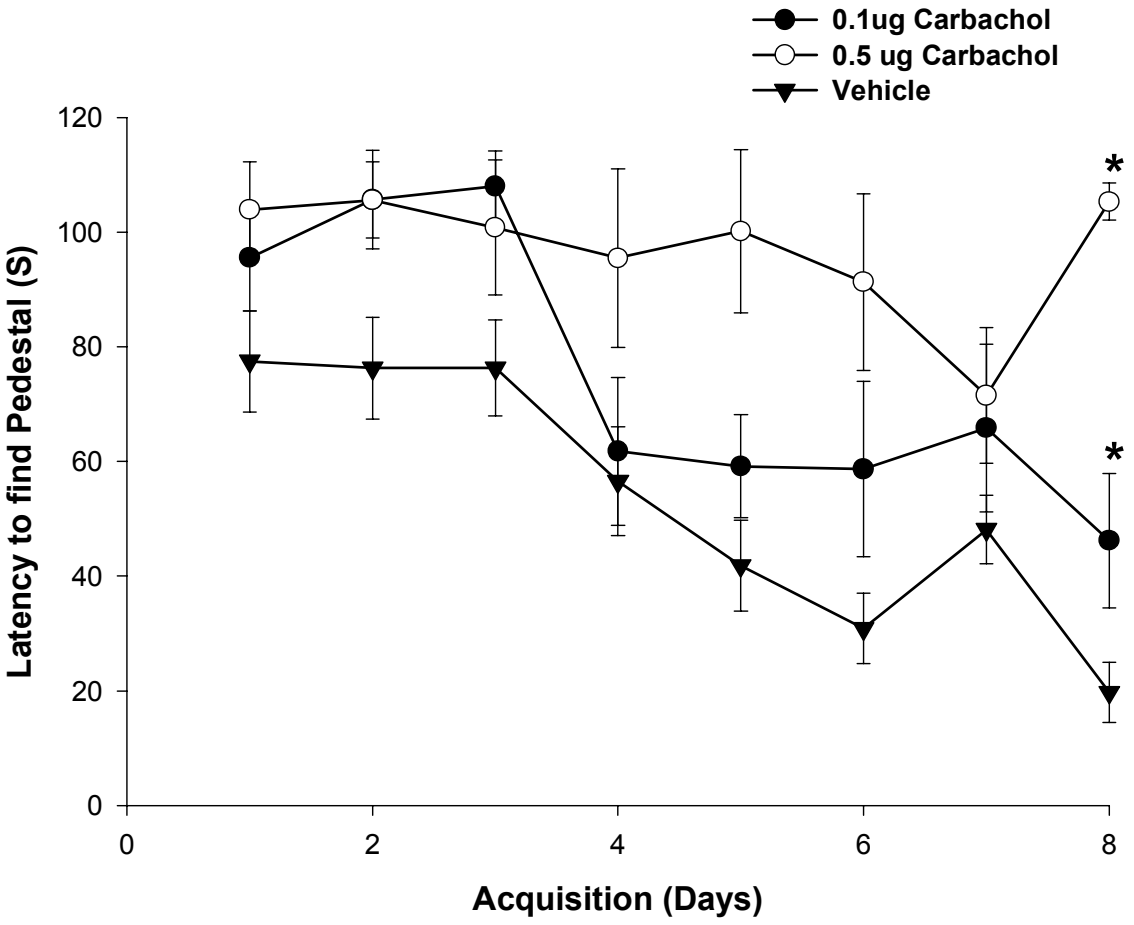

10 nmol divalinal
0.25 μg carbachol + 10 nmol divalinal
0.5 μg carbachol + 10 nmol divalinal
Vehicle
Latency to Find Pedestal (sec)
120
100
80
60
40
20
0
0
2
4
6
8
Acquisition (days)
*

Figure 4


0.1ug Carbachol
0.5 ug Carbachol
Vehicle
Latency to find Pedestal (S)
120
100
80
60
40
20
0
0
2
4
6
8
Acquisition (Days)
*
*

CHAPTER THREE

THE ROLE OF NORLEUCINE^{1}-ANGIV (NLE^{1}-ANGIV) IN SPATIAL MEMORY IN NUCLEUS BASALIS MAGNOCELLULARIS (NBM) SCOPOLAMINE-INDUCED DEFICITS IN RATS

THE ROLE OF NORLEUCINE[1]-ANGIV (NLE[1]-ANGIV) IN SPATIAL MEMORY IN NUCLEUS BASALIS MAGNOCELLULARIS (NBM) SCOPOLAMINE-INDUCED DEFICITS IN RATS

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Abstract

The brain AT_4 and cholinergic systems play a pivotal role in learning and memory. Many studies have investigated the cognitive enhancing properties of both systems. The cholinergic system has received the most attention and appears to contribute to normal and abnormal cognitive functioning. For example, one of the best known cognitive disorders, Alzheimer's disease (AD), is treated with cholinergic-directed drugs, while post-mortem studies of AD patient brains show neurodegenerative devastation in cholinergic areas of the brain. Recent studies have suggested that potentiation of cholinergic transmission may be a mechanism by which the AngIV/AT_4 receptor system enhances cognition (Lee, Chai et al. 2001; Olson, Olson et al. 2004), however, neural pathways between cognitive structures has never been explored between the two systems. Since the Nucleus Basilis Magnocellularis/Meynert (in primates) (NBM) is a main source of cholinergic innervation to major cognitive areas of the brain, this site was chosen to investigate the interaction of the two systems. Previous findings from our laboratory have consistently found facilitatory activity of AT_4 receptor agonists in various cognitive impairment models. In contrast, the present study found that ICV Nle^1-AngIV did not attenuate water maze acquisition impairments produced by intra-NBM scopolamine. These results suggest a dominant role for the cholinergic system of the NBM in spatial memory acquisition. It is evident that a complex relationship exists between AT_4 and cholinergic receptors in the NBM and the hippocampus. Possible explanations for these findings are discussed.

Abbreviations

ACh, acetylcholine; aCSF, artificial cerebrospinal fluid; AngIV, angiotensin IV; ICV, intracerebroventricular; NBM, Nucleus Basalis Magnocellularis (Rat)/Meynert (human)

1. Introduction

AngIV is a hexapeptide which through activation of the AT_4 receptor mediates various functions in the central nervous system (Wright, Krebs et al. 1995; Wright, Stubley et al. 1999). AT_4 agonists have been found to facilitate learning and memory in many rat models (Wright, Clemens et al. 1996; Pederson, Harding et al. 1998; Pederson, Krishnan et al. 2001; Albiston, Pederson et al. 2004; Lee, Albiston et al. 2004; Olson, Olson et al. 2004; Meighan, Meighan et al. 2007) and to alter long-term potentiation, a cellular model of learning and memory (Kramar, Armstrong et al. 2001; Wayner, Armstrong et al. 2001; Wright, Kramar et al. 2003). The localization of AT_4 receptors further supports these findings since receptors are densely populated in areas well-established to be associated with cognitive processing (Wright, Miller-Wing et al. 1993; Harding, Wright et al. 1994).

Much like the brain AT_4 system, the brain cholinergic system has many similar traits. Agonists of AT_4 and cholinergic receptor subtypes, muscarinic Acetylcholine receptors (mAChR) and nicotinic (nAChR) are facilitatory to learning and memory in both human and animal cognitive tasks (Dennes and Barnes 1993; Newhouse, Potter et al. 2004; Buccafusco, Letchworth et al. 2005; De-Mello, Souza-Junior et al. 2005). Conversely, antagonists of both the AT_4 receptor (Wright, Stubley et al. 1999) and AChR's are capable of impairing learning and memory in animals and humans (Flood and Cherkin 1986; Dickson and Vanderwolf 1990; Dennes and Barnes 1993; Ebert and Kirch 1998; Buccafusco, Letchworth et al. 2005; De-Mello, Souza-Junior et al. 2005).

An interaction between these systems is evident; however the specifics are only now being explored. For example, our laboratory, as well as others, have found that AT_4 agonists delivered into the ventricles can overcome either MAChR or NAChR blockade-induced deficits in the water maze (Pederson, Harding et al. 1998; Pederson, Krishnan et al. 2001; Olson, Olson et al. 2004) and passive avoidance tasks (Braszko, Kupryszewski et al. 1988; Albiston, Pederson et al. 2004). Furthermore, nicotine (nAChR ag) administered into the Nucleus Basalis Magnocellularis/Meynert (NBM) can reverse intra-NBM AT_4 – blockade produced amnesic properties (unpublished results).

The following study further explored the interaction of these systems in the basal forebrain, specifically the NBM. This site was chosen because: 1) it contains both AT_4 receptors and cholinergic receptors, 2) normal functioning of this area appears necessary for cognitive processing, and 3) the investigators desired to further explore previous findings of an interaction between the two systems in this area.

2. Methods

2.1 Animals and surgery

Male Sprague-Dawley rats (90 -120 days old) weighing 350-550 g were housed in an AAALAC-approved vivarium maintained at 22 ± 1 °C, with a 12:12 h light cycle initiated at 07:00 h. All animals were allowed ad libitum access to normal rat chow (Harland Teklad Rodent Diet, Madison, WI) and tap water throughout the experiment. The rats were anesthetized with Equi-thesin (0.3 ml/100 g ip),

and Lidocaine (Phoenix Pharmaceutical, Inc. St.Joseph, MO) was used as a local anesthetic. Topical Betadine (Fabrique Par H&P Industries Inc. Mukwonago, WI) was applied following surgery to prevent post surgical infection. All rats were fitted with a chronic bilateral cerebral NBM and right-side intracerebroventricular (ICV) guide cannula (PE-60, Clay Adams. Sparks, MD, length = 2.5 cm) by sterotaxic surgery positioned above both NBM and the right ventricle. Coordinates for the NBM were 1.8 mm posterior to bregma and +/- 3.0 mm lateral from the midline. Coordinates for the ICV guide cannula were 1.0 mm posterior to bregma and 1.5 mm lateral from the midline. A heat bulge, positioned 2.5 mm from the tip of the cannula, controlled the depth of penetration into the skull. After stereotaxic placement the cannulas were secured with stainless steel skull screws and dental cement. Four days following surgery, animals were handled for approximately 5 min per day for the last 3 days of post-surgical recovery. Animals were allowed 7-10 days of post-operative recovery at which time behavioral testing commenced. Correct cannula placement was verified postmortem with an injection of green dye infusion into each cannula followed by histology upon termination of all behavioral testing.

2.2 Compounds

Nle^{1}-AngIV (Pacific Northwest Biotechnology, Pullman, WA) and scopolamine (Sigma-Aldrich Co., St. Louis, MO) were dissolved in aCSF as the vehicle.

Nle^{1}-AngIV was administered at a dose of 50 pmol/μl aCSF icv (2 μl total volume) and scopolamine at a dose of 35 nmol/1 μl bilaterally into each NBM (1μl total volume/NBM).

All infusions were administered via 10 μl Hamilton microsyringes, attached to PE 20 tubing, hand-delivered over a period of 30 s. The injector consisted of 29-gauge stainless steel tubing, 30.8 cm in length (penetrating 6.8 mm ventral to dura) for all NBM injections, and 2.65 cm in length (penetrating 4 mm ventral to dura) for all ICV injections.

2.3 Circular water maze

The water maze apparatus was a circular black tank (diameter: 1.6 m; height 0.6 m), filled with water kept at a constant temperature of 26-28 °C. The maze was partitioned into four equal quadrants of NW, NE, SW and SE. Animals began the trial at a different entry point, facing the wall of the tank each trial. The entry points were denoted as one of the four quadrant corners (i.e. N, S, E and W) and were randomly assigned per trial. Extra-maze spatial cues consisted of different colored cardboard shapes (circles, squares and triangles) on three of the four walls in the testing room.

Water maze trials were run in 2 different phases. The pre-training phase consisted of 2 days, 5 trials per day. The animals were trained to escape the water by swimming to and climbing upon a visible pedestal (~1cm above water) placed in the center of the tank (without administration of any drug treatment). The rats were placed in the water at counter-balanced entry points. Latency

(sec) to find the pedestal was recorded; each rat was allowed a maximum of 120 s per trial to locate the pedestal. Once the animal found the pedestal, it was allowed a 30 sec rest period. If the rat failed to locate the pedestal, the experimenter placed the animal on the pedestal and allowed for the 30 sec rest period. Immediately following the rest period the next trial ensued. In phase two, the acquisition trials consisted of five consecutive days, with five trials conducted per day. The trials required the rat to locate a hidden pedestal (submerged 2 cm below the water line), which was placed in one of the four quadrants and remained fixed for the duration of acquisition training. The rat was allowed 120 s per trial to locate the pedestal. Once the animal found the pedestal, it was allowed a 30 s rest period. If the rat failed to locate the pedestal, the experimenter placed the animal on the pedestal for the 30 s rest period. Immediately following the rest period the next trial ensued. In both phases swimming path was analyzed by a computer video tracking system (Chromotrack, San Diego Instruments,San Diego, CA). The computer recorded total swim latency upon locating the pedestal, in addition to total swim distance per trial. Swim speed was calculated by dividing the swim distance by the swim latency. Following the last daily trial, all animals were immediately towel dried, placed in its home cage and warmed under a heat lamp for 10-15 min until dry.

2.4 Histology

Correct cannula placement was verified postmortem with an injection of 2 μl of green dye. A lethal injection of Equithesin (Chloral hydrate and

Pentobarbital, i.p.) was administered, followed by removal of the brain. Brain tissue was post-fixed in formalin (9 %) and sectioned at 40 µm using a freezing microtome to assess proper cannula location.

2.5 Statistics

Differences among groups on latency and swim speed to find the pedestal each day (mean of 5 trials each day) during the acquisition trials (phase two) were assessed using a two-way ANOVA, with groups being between subjects and days of acquisition within subjects. The same data were assessed for differences amongst groups during pre-training trials, (phase 1) and the final day of acquisition, (day 7) except one-way ANOVAs were performed.

Data were analyzed by analysis of variance (ANOVA). Significance levels were set at $P<0.05$. Post-hoc analyses were performed using Tukey's HSD ($P<0.05$) in order to evaluate significant differences. Data are presented as mean ± S.E.M.

3. RESULTS

A 4 (groups) X 5 (days) ANOVA found a main effect of groups in latency to reach the pedestal ($F(3,19)=6.319$, $p<.05$), in addition to a main effect of number of trials completed ($F(6,18) =119.94$,$p<.05$). However, no interaction effect. Tukeys HSD post-hoc analysis indicated that the Scopolamine and Scopolamine/ Nle^1-AngIV cocktail treated rats were significantly different from both the vehicle group and Nle^1-AngIV treated rats ($p<.05$); Nle^1-AngIV treatment was not different from control. (See figure 1). A one-way ANOVA

performed on the last test day (day 7) indicated that only the scopolamine treatment group latencies were different from controls ($F(3,22)=5.07$, $P<.01$). This suggests a weak facilitatory effect of Nle^1-AngIV on scopolamine-induced impairments; however the scopolamine/Nle^{1}AngIV group was not significantly different from the scopolamine group. It should be noted that this effect was only seen on the final day of training and could be an insignificant anomaly in the data.

Insert Fig 1 here

---

4. DISCUSSION

The present study found that Nle^1-AngIV was unable to overcome intra-NBM scopolamine-induced impairments in the water maze task. Analysis of the data showed little if any reversal effect by the AT_4 agonist in scopolamine treated rats. There was a small difference between the controls and the Nle^1-AngIV/scopolamine treated rats on the final day of acquisition (day 7); however there was no difference between the Nle^1-AngIV/scopolamine group with the scopolamine only treated rats. These results indicate a weak effect of Nle^1-AngIV, however, this effect is minimal and was only seen on day 7. These data are somewhat puzzling since icv Nle^1-AngIV is capable of overcoming both scopolamine- and mecamylamine-induced deficits (Pederson, Harding et al.

1998; Pederson, Krishnan et al. 2001; Olson, Olson et al. 2004). The difference between this study and previous studies investigating the faciliatory activity of Nle^1-AngIV was that in the current study the cholinergic antagonists were administered directly into the NBM, instead of into the lateral ventricle.

The NBM is densely populated with cholinergic receptors and efferent projections to the mantle of the cortex. This area of the basal forebrain has been found to be exceptionally important in cognitive processing, and appears to be involved in the pathophysiology of AD. The exact mechanisms and interacting neurotransmitter systems are still under investigation; however our laboratory has found that blocking either cholinergic or AT_4 receptors in the NBM severely impairs water maze acquisition. This implies functional properties of these systems in the NBM with regards to spatial learning. Recently, our laboratory found that nicotine infused directly into the NBM can overcome AT_4 antagonist induced acquisition impairments in the water maze (Wilson, unpublished). To better understand the interaction between the AT_4 and cholinergic systems in cognitive areas of the brain, the present study investigated the capability of icv infusions of Nle^1AngIV to overcome intra-NBM induced cholinergic receptor blockade-deficits in the water maze.

These results indicate that activating AT_4 receptors via icv (presumably through activation of hippocampal circuits) were weak, but promising in overcoming the NBM-induced cholinergic impairments. Nle^1-AngIV administered directly into the NBM was also weak in attenuating these scopolamine-induced effects (data not shown). There are several possible

explanations why these results differ from previous findings regarding Nle^{1}AngIV's robust nootropic activity. One possible explanation concerns the ratio of cholinergic vs AT_4 receptors in the NBM. It is generally agreed that the primary system mediating learning in this area is the cholinergic system (Pepeu and Marconcini Pepeu 1994; Muir 1997; Mesulam 1998; Lucas-Meunier, Fossier et al. 2003). However, histological and autoradiographic findings indicate the presence of a less dense population of AT_4 receptors, along with many other neurotransmitter receptor systems (Wright, Miller-Wing et al. 1993; Moeller, Paxinos et al. 1996; Semba 2000). Thus, it is likely that blocking muscarinic receptors with scopolamine, which are abundant and functionally important in this area, cannot easily be compensated for by increasing AT_4 receptor activation in the NBM or icv. Consistent with this notion is the finding that AT_4 receptor blockade with divalinal in the NBM impairs acquisition in the water maze and infusion of nicotine into the NBM can overcome this impairment. Thus, the explanation for the present findings may lie in the ratio of receptors present in the NBM, especially since blocking either system can impair, but the cholinergic system can overcome the AT_4 blocked-effects, whereas Nle^{1}AngIV effects are promising, but not confirmed. Furthermore, although both systems are present and functionally important in the NBM with respect to cognitive processing, the cholinergic system appears to be functionally dominant.

Other potential explanations for the weakness of the Nle^1-AngIV effects following NBM infusions include the doses administered. Specifically, this is a

very small structure and no studies have looked at varying dose ranges of AT_4 compounds into this brain region: previously this class of neuropeptides has only been administered into the ventricles(Pederson, Harding et al. 1998; Pederson, Krishnan et al. 2001; Lee, Albiston et al. 2004; Olson, Olson et al. 2004). Additionally, studies in our laboratory have shown antagonist activity of Nle^1-AngIV at excess doses, portraying the characteristic inverted U-shaped curve of agonist activity followed by antagonist activity at the receptor (Harding, personal communication).

It is likely that the NBM is a downstream target from the hippocampus with regard to learning and memory processing. In this way the hippocampus sends AT_4 modulatory projections (presynaptic facilitation) to the NBM to facilitate transfer of information to storage sites in the cortex. AngIV binds at presynaptic receptors on cholinergic neuron and through regrograde nitric oxide messaging causes the release of ACh at presynatpic terminals within the NBM (Lee, Albiston et al. 2004). This structure in turn sends cholinergic projections to the mantle of the cortex for further memory processing and storage. The notion that nitric oxide is a retrograde messenger is supported by data collected in our laboratory showing that AngIV and Nle^1-AngIV effects on cerebral blood flow depend on the synthesis and release of nitric oxide (Kramar, Krishnan et al. 1998). Also, evidence of afferent projections to the NBM indicate that this structure is a major relay station between limbic structures and the neocortex (Mesulam, Mufson et al. 1983). Thus, it is conceivable that the hippocampus relies on the NBM for the full consolidation of information. Incorporating this possibility into the

interpretation of the current results we found that icv Nle^1-AngIV was weak in overcoming scopolamine blockade within the NBM. Putting this into perspective, icv Nle^{1}AngIV is presumably having the most effect on the hippocampus, and very little effect on the NBM, thus AT_4 receptor activity would be upstream from the cholinergic blockade in the NBM. Also, in considering Nle^1-AngIV's weak effectiveness in the NBM; this may be due to the comparatively smaller number of AT_4 vs ACh receptors in this area. AT_4 receptors are denser in the hippocampus than in the NBM, the opposite being true of the cholinergic receptor system.

It is apparent from these findings that the relationship between the AT_4 system and cholinergic system is extremely complex. It appears that the AT_4 system plays a modulatory role, and that in the NBM the cholinergic system is dominant. Further research needs to be conducted on these systems to confidently elucidate their exact interaction. Continuing this research could provide insight for future pharmacological cocktails in the treatment of cognitive impairments.

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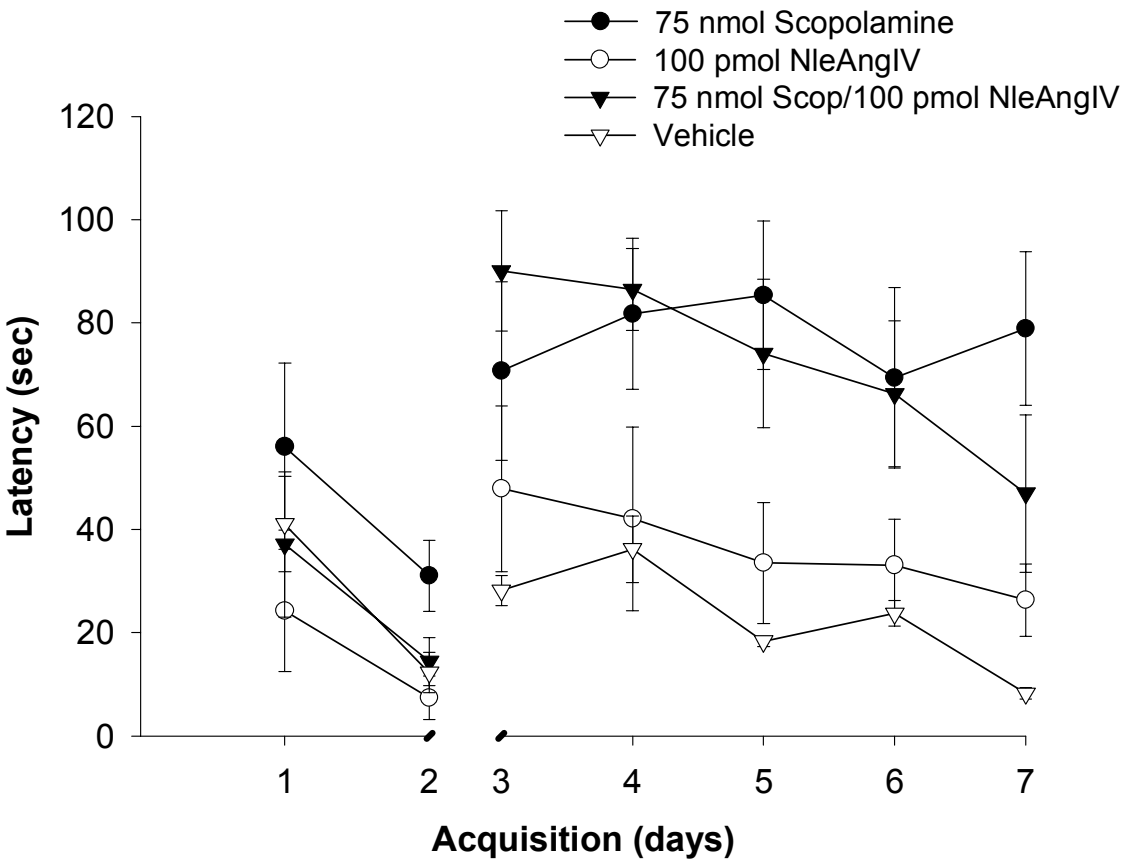
Fig. 1. Effects of administration of 100 pmol Nle^{1}-AngIV (n=8),75 nmol scopolamine (n=8), 100 pmol Nle^{1}-AngIV/75 nmol scopolamine (n=8), or vehicle (aCSF) (n=8) on spatial learning. Nle^{1}-AngIV was administered icv; scopolamine was administered directly into the NBM. The data are expressed as mean ± SEM (averaged over 5 trials per session).

Fig 1


75 nmol Scopolamine
100 pmol NleAngIV
75 nmol Scop/100 pmol NleAngIV
Vehicle
Latency (sec)
120
100
80
60
40
20
0
1
2
3
4
5
6
7
Acquisition (days)

CHAPTER FOUR

GENERAL DISCUSSION

GENERAL DISCUSSION

These experiments were designed to reveal clues regarding the relationship and interaction between the AT_4 and cholinergic systems in cognitive processing. In review of previous studies investigating this association, the cholinergic antagonist, scopolamine consistently produces learning and memory deficits (given peripherally or centrally) (Flood and Cherkin 1986; Dickson and Vanderwolf 1990; Ebert and Kirch 1998), whereas icv administered AT_4 agonists are beneficial in reversing deficits seen in these compromised animals, often inducing equivalent performances to controls (Erfurth and Holmes 1995; Pederson, Harding et al. 1998; Pederson, Krishnan et al. 2001; Lee, Albiston et al. 2004; Olson, Olson et al. 2004). The nucleus basalis of meynert (NBM) is an important structure involved in learning and memory processing. This area of the brain has been consistently found to undergo severe neurodegeneration in Alzheimer's patients (Muir 1997; Gsell, Jungkunz et al. 2004; Teipel, Flatz et al. 2005), suggesting its role in the cognitive impairments that characterize this disease. Additional support for the significance of the NBM in cognitive impairments is seen in animal models in which lesioning of this area produces profound impairments in a variety of learning and memory tasks (Olton 1990; Page, Everitt et al. 1991; Patel and Tariot 1991; Pepeu and Marconcini Pepeu 1994; Baxter, Bucci et al. 1995; Li, An et al. 1998; Tian, Lin et al. 2004). Degeneration of the cholinergic system has been hypothesized to play a significant role in Alzheimer's disease (AD) and other dementias (Mesulam 1998;

Erickson and Barnes 2003; Gsell, Jungkunz et al. 2004). Interestingly, the NBM is a major cholinergic area, containing a high number of cholinergic cell bodies in addition to sending widespread projections to the neocortex (Mesulam, Mufson et al. 1983; Decker 1987; Lucas-Meunier, Fossier et al. 2003). Currently, only five drugs are approved for use in the treatment of the cognitive deficits in AD; 4 of the 5 act to enhance the cholinergic system (the newest drug acts on the NMDA receptor system). However, these drugs are only mildly beneficial, and their effectiveness is limited to the early stages of the disease. It is apparent that other neural systems are involved in the devastation of this disorder, thus, further understanding of the neural relationships underlying normal and abnormal cognitive processing is imperative for the development of novel therapeutics in treating cognitive associated ailments. Therefore, the overall objectives of these studies were designed to investigate the following: 1) Is the AT_4 system present in the NBM, and if so, does it play a role in cognitive processing? 2) What is the role of the AT_4 and cholinergic systems in the NBM, and do these systems interact or are they separate autonomous systems?

Summary of Findings

Divalinal-AngIV, an AT_4 receptor antagonist, produced profound deficits in performance in the circular water maze. Pre-treatment with nicotine completely reversed these divalinal-AngIV induced impairments. In contrast, carbachol, a muscarinic receptor agonist, did not affect this impaired acquisition, and at higher doses appeared to exaggerate the divalinal induced-deficits. Similar to the AT_4

antagonist, both scopolamine and mecamylamine (muscarinic and nicotinic receptor antagonists, respectively), prevented acquisition of the water maze task. Based on these results, it appears that blocking any one of these systems results in impaired spatial learning, while activating the nicotinic receptor system counteracts the effects of AT_4 receptor blockade. These findings support previous studies demonstrating a functional role for both the cholinergic and AT_4 receptor systems in spatial learning, and indicate for the first time a functional role for the AngIV/AT_4 receptor system in the NBM.

To further investigate this functional role, the AT_4 agonist, Nle^1-AngIV (icv) was assessed for its ability to overcome cholinergic blockade in the NBM.. Surprisingly, results found that icv Nle^1-AngIV was weak in reversing intra-NBM scopolamine-induced impairments in the water maze task. This result indicates that activating AT_4 receptors via icv (presumably mostly through activation of hippocampal circuits) is not enough to overcome the cholinergic blockade in the NBM. In addition, Nle^1-AngIV directly into the NBM was also weak in attenuating these scopolamine-induced effects (data not shown). One possible explanation takes into account ratio comparisons of these receptor populations in the NBM. It appears that blocking muscarinic receptors, which are abundant and functionally important in this area, cannot be compensated for by increasing AT_4 receptor activation in the NBM or via icv administration. This is congruent with our findings showing AT_4 receptor blockade (with divalinal) in the NBM impairs acquisition in the WM; however, introduction of the nicotinic cholinergic agonist (nicotine) is fully capable of overcoming the behavioral impairments produced by

the divalinal. The discrepancy in these findings could be explained by differences in neurotransmitter receptor system ratios in the NBM, especially since blocking either system can impair, but only the cholinergic system can overcome the AT_4 blocked-effects. Furthermore, it can be said that both systems are present and functionally important in the NBM with respect to cognitive processing. However, collectively these results indicate that in the pharmacological manipulation of these two systems, the NBM is superior in mediating spatial learning when compared to structures accessible via ICV infusions. These findings also imply that the cholinergic system is functionally dominant in the NBM whereas the AT_4 system is most important in modulating activity of the cholinergic system.

In conclusion, the AT_4 system appears to play a significant role in mediating cholinergic processing in the NBM. Firstly, results showed that blocking any of these three systems in the NBM severely impairs spatial learning. secondly, activating cholinergic systems in the NBM can overcome AT_4 antagonist effects, however, AT_4 facilitation in the hippocampus (via icv administration) is unable to override cholinergic blockade in the NBM. It is evident that additional studies need to be conducted to completely understand the complex interaction of these systems in cognitive areas of the brain.

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