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Selective Lesion of Cholinergic Neurons of the Septal-Hippocampal Tract: Memory and Learning

Nicholas Fitz

SELECTIVE LESION OF CHOLINERGIC NEURONS OF THE SEPTAL HIPPOCAMPAL TRACT: MEMORY AND LEARNING

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By

Nicholas Francis Fitz

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APPROVED

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David A. Johnson, Ph.D., Chair of Dissertation Committee
Associate Professor of Pharmacology
Director of Graduate Studies
Graduate School of Pharmaceutical Sciences, Duquesne University
Pittsburgh, PA

APPROVED

_____

Paula A. Witt-Enderby, Ph.D.
Professor of Pharmacology
Graduate School of Pharmaceutical Sciences, Duquesne University
Pittsburgh, PA

APPROVED

_____

Christopher K. Surratt, Ph.D.
Associate Professor of Pharmacology
Division Head of Pharmaceutical Sciences
Graduate School of Pharmaceutical Sciences, Duquesne University
Pittsburgh, PA

APPROVED

_____

Robert B. Gibbs, Ph.D.
Professor of Pharmaceutical Sciences
School of Pharmacy, University of Pittsburgh,
Pittsburgh, PA

APPROVED

_____

Michael E. Rhodes, Ph.D.,
Assistant Professor of Biology
School of Natural Sciences, Mathematics and Computing,
Saint Vincent College, Latrobe, PA

APPROVED

_____

J. Douglas Bricker, Ph.D.
Dean, Mylan School of Pharmacy
Associate Professor of Pharmacology-Toxicology
Graduate School of Pharmaceutical Sciences, Duquesne University
Pittsburgh, PA

ABSTRACT

SELECTIVE LESION OF CHOLINERGIC NEURONS OF THE SEPTAL HIPPOCAMPAL TRACT: MEMORY AND LEARNING

By

Nicholas Fitz

May 2009

Dissertation supervised by David A. Johnson Ph.D.

It is hypothesized that the loss of cholinergic function in the medial septum (MS), observed early in many forms of dementia, contributes to memory losses characterized in these diseases. The studies of this dissertation examined whether the selective loss of cholinergic neurons in the MS impairs acquisition of a delayed matching-to-position (DMP) spatial memory task. The results suggest a significant contribution of MS cholinergic neurons in acquisition of the DMP task. Specifically, 192 IgG-saporin SAP lesioned rats acquired the task at a slower rate and required more days to reach criterion. The results also suggest that male rats typically adopt a consistent turning strategy early in the training process, which is independent of extra-maze cues. For animals to reach criterion, an alternative learning strategy was adopted; one dependent on extra-maze cues. Cholinergic lesion of the MS resulted in a greater reliance on a consistent turning strategy, which accounted for the slower rate of acquisition of the DMP task.

Steroid sulfatase inhibitors increase whole brain DHEAS levels, enhance ACh release in the hippocampus, and enhance memory. The present study also investigated the cognitive effects of sulfatase inhibition in SAP lesioned animals. Steroid sulfatase inhibition further impaired acquisition of the DMP task in SAP lesioned rats while having no effect on cholinergically intact animals. Since DHEAS displayed memory enhancing properties in rodents, we also investigated the effects of DHEAS administration on MS SAP lesioned animals. DHEAS treatment had no significant effect on the acquisition of the DMP task in the SAP treated or control animals.

The final study of this dissertation examined the effect of arousal on DMP performance in SAP lesion of the medial septum. Arousal, induced by the IP injection of saline, decreased the number of days SAP lesioned rats needed to reach criterion and also improved the rate of acquisition. The results suggest that a mild aversive stimulus can attenuate cognitive deficits caused by MS cholinergic lesions.

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LIST OF ABBREVIATIONS

ACh = Acetylcholine

AChE = Acetylcholinesterase

aCSF = Artificial cerebral spinal fluid

AD = Alzheimer's disease

AMPA = α-amino-3hydroxy-5-methyl-isoxazole-4-proprionic acid

ChAT = Choline acetyltransferase

CNS = Central nervous system

CoA = Coenzyme A

CPP = Conditioned place preference

DBB = Diagonal band of broca

DHEA = Dehydroepiandrosterone

DHEAS = Dehydroepiandrosterone sulfate

DMP = Delayed match-to-position

DU-14 = (P-o-sulfamoyl)-N-tetradeconoyl tyramine

GABA = γ- aminobutyric acid

HPA = Hypothalamic-pituitary-adrenal

MS = Medial septum

NBM = Nucleus basalis magnocellularis

NMDA = N-methyl-D-aspartate

SAP = 192-IgG-saporin

I. INTRODUCTION

A. Literature Review

1. Dementia:

Dementia is a syndrome characterized by neuropsychological, neuropsychiatric, and neurologic manifestations. Particularly affected functions include memory, attention, language, and problem solving. Symptoms of dementia can be reversible or irreversible. Reversible causes of dementia include hypothyroidism, Vitamin A, B_1, or B_{12} deficiency, tumors, normal pressure hydrocephalus, or syphilis; but these only account for less than 10% of clinical cases. Irreversible dementias include Alzheimer's disease, vascular dementia, alcohol induced persisting dementia, and frontotemporal lobar degeneration. There is an estimated 24 million people worldwide with clinical symptoms of dementia, and this number is projected to increase to 81 million by 2040 (1).

Alzheimer's disease (AD) is the most common type of dementia in the elderly, affecting almost half of all patients with dementia. More that 5 million Americans are estimated to have AD and it is projected that 14.3 million Americans will develop the disease by 2050; a 350% increase from 2000 (2). Among patients 65 years of age, 2-3% display symptoms of the disease, while nearly 50% of patients 85 years of age or older have symptoms of AD and an even greater number display some of the pathological hallmarks of the disease without presenting the symptoms. Statistics show that for every five years a person lives past the age of 65, the probability of developing the disease doubles (3). As global life expectancy rises, patients over the age of 85 are the fastest growing segment of the AD population. Therefore, advanced aging is a primary risk factor for the disease. Clinicians who diagnose and treat patients with AD generally

recognize that the prevalence of AD is higher in women (3). Possibly due to the relative lack of a protective factor, such as the estrogen deficiency of postmenopausal women, which may increase the vulnerability to AD pathology.

For most patients diagnosed with the disease, increased impairments in learning and short-term memory will lead to a diagnosis of dementia, while small populations may also display signs of language and visual-spatial difficulties (4). Memory problems do not affect all memory capacities equally, with older memories of the patient's life, facts learned, and implicit memory commonly spared (5-6). These initial symptoms progress from simple forgetfulness and the inability to navigate simple tasks, to more persistent losses of short-term memory and difficulty navigating through familiar areas, such as one's own home. As the disease progresses to the moderate stage of memory loss, patients fail to recognize familiar objects and persons (4). During this stage of the disease, patients may also show changes in behavior, such as violent outbursts or excessive passivity without prior history of such behaviors. In late stages of the disease, patients lose the ability to perform even simple tasks independently. Patients become incontinent of bladder and bowel, and/or lose the ability to walk or eat without assistance. In late stages of AD, language becomes disorganized and then lost altogether. Eventually, the ability to swallow food or drink is lost, ultimately leading to death (4). In 2004, with 65,829 deaths, AD was the seventh leading cause of death in the United States (7). The mean duration of the disease is 8.5 years between onset of the clinical symptoms and death. At a cost of over $100 billion per year, AD is the third most costly disease in the United States, after heart disease and cancer. Direct and indirect costs for long term care for an Alzheimer's patient averages $77,500 per year (8).

Regions associated with higher brain function, particularly the neocortex and hippocampus, are most affected by the pathology of AD. Pathological changes include extracellular deposits of β-amyloid, a proteolytic byproduct of the transmembrane protein amyloid precursor protein. Although β-amyloid monomers are soluble and harmless, they undergo a conformational change at high concentrations to form a β-sheet rich tertiary structure that aggregates to form amyloid fibrils. These amyloid fibrils are deposited extracellularly, forming dense senile plaques. Another pathological characteristic of AD is abnormal aggregation of tau protein, a microtubule associated protein expressed in neurons that normally acts to stabilize microtubules in the cytoskeleton. Like most microtubule-associated proteins, tau is normally regulated by phosphorylation. However in AD patients, hyperphosphorylated tau accumulates as paired helical filaments that form intracellular neurofibrillary tangles and dystrophic neurons associated with amyloid plaques (9). There is also a significant loss of neuronal synapses and pyramidal neurons within the brain regions associated with higher cognitive function. The cholinergic basal forebrain neurons are one of the most sensitive targets of degenerative processes occurring in patients with AD (10-11).

Three major competing hypotheses exist to explain the cause of Alzheimer's disease. The cholinergic hypothesis of AD proposes that degeneration of cholinergic neurons in the basal forebrain and the associated loss of cholinergic neurotransmission in the cerebral cortex and hippocampus contributes significantly to the deterioration of cognitive function seen in AD patients (12). In support of this hypothesis, postmortem AD brains are characterized by neuronal loss and neurofibrillary tangle formation in regions of the neocortex and hippocampus, primarily affecting pyramidal neurons and

their synapses (13-14). Furthermore, neurotransmitter specific projections to the cortex and hippocampus are affected by neurodegenerative processes, including cholinergic nucleus basalis and medial septum projections (11). For example, AD affected brains have an almost 80% reduction in cholinergic neurons of the nucleus basalis compared to age-matched control brains. Biochemical examination of biopsy tissues from patients with AD 3.5 years following the onset of symptoms indicate selective cholinergic pathologies occur early in the course of the disease (15). Specifically, presynaptic markers of cholinergic activity are greatly reduced. Reduction of choline acetyltransferase (ChAT) activity and acetylcholine (ACh) synthesis were strongly correlated with the degree of cognitive impairment in AD patients (15-18). Subsequent findings of reduced choline high-affinity uptake, impaired ACh release, deficits in expression of nicotinic and muscaric cholinergic receptors, and loss of cholinergic soma from the nucleus basalis confirmed a substantial presynaptic deficit (reviewed in 19). Cholinergic effects have also been proposed to initiate large-scale β-amyloid aggregation leading to generalized inflammation (20). In further support of the cholinergic hypothesis for AD, anticholinergic drugs impair learning and memory in a way that was similar to that observed with AD (21). Currently, despite these findings, the cholinergic hypothesis is not widely accepted. Even if the disruption of the cholinergic system is not the first stage of the pathogenesis of AD, it is still a major event that is involved in the loss of cognitive function, a key hallmark of the disease.

The other hypotheses for the pathogenesis of AD focus on the effects of the misfolded and aggregated proteins β-amyloid and tau. One hypothesis states that tau hyperphosphorylation initiates the disease cascade, while another hypothesis states that β-

amyloid deposits are the initiating factor. The tau hypothesis is supported by the observation that the deposition of amyloid plaques do not correlate well with the neuronal loss observed in AD (22). The cloning of the gene encoding the β-amyloid precursor protein and its localization to chromosome 21, coupled with the earlier observation that Down's syndrome (Trisomy 21) mimics the neuropathology of AD, lead to the hypothesis that β-amyloid accumulation is the primary event in AD pathogenesis. The formulation of the amyloid hypothesis points to the cytotoxicity of mature aggregated amyloid fibrils, which are thought to disrupt calcium homeostasis and thus induce apoptosis (23-24).

There is currently no cure for AD and the medications currently available offer only small symptomatic benefit for some patients, but do not slow disease progression. Approaches for treating AD disease have focused on augmenting cholinergic function, since decreased central cholinergic activity appears to be an important component of the pathology of memory and attentional deficits associated with AD. Drug therapies can enhance cholinergic function through several different mechanisms. Acetylcholinesterase (AChE) inhibitors (tacrine, donepezil, galantamine, rivastigmine) enhance cholinergic function by inhibiting the hydrolysis of ACh and therefore, heighten the concentration of ACh at the post-synaptic receptor sites. Unfortunately, AChE inhibitors, aside from being marginally effective, are also limited by a lack of specificity. Moreover, they do not alter the course of the underlying disease process.

2. Learning and Memory

Learning is the cognitive process of acquiring skills, knowledge, and understanding from study, instruction, or experience that help guide long term behaviors. During animal behavioral testing, learning occurs when a new response becomes associated with a particular stimulus is termed associative learning. Associative learning occurs through classical and instrumental conditioning. The typical procedure for inducing classical conditioning involves presentation of light or sound followed by administration of food. During classical conditioning animals learn to associate the light with food. Habituation is the waning of an animal's behavioral response to a stimulus. Habituation is usually considered a form of learning which involves the elimination of behaviors that are not needed by the animal. Habituation is separated from most other forms of decreased response on the basis that the change in behavior is permanent; habituated animals do not resume earlier reaction to the stimulus after a period of non-stimulus. Learning would be extremely inefficient if humans had to rely completely on classical and instrumental conditioning. Unlike in animals, humans can acquire information through cognitive learning. Cognitive learning is defined as the acquisition of knowledge and skill by mental processes, observation, instruction, and imitation of behavior. In cognitive learning, the individual learns by listening, watching, touching, reading, or experiencing.

Memory is defined as the mental faculty of retaining and recalling past experiences (25). From an informational processing standpoint there are three stages in memory formation: encoding, storage, and retrieval. Encoding is the processing of sensory input into memory. During memory encoding, information is processed about

space, time, and frequency. Storage is the organization of the encoded information that is retained in sensory, short-term, or long-term memory. Retrieval of memory involves the recall of stored information and can be classified into three different categories: free recall (no clues given to assist retrieval), serial recall (items recalled in a particular order), and cued recall (clues given to assist retrieval) (26). During retrieval of memories neurons are activated and fired in the same pattern as during the encoding process.

Memory can be generically classified on the bases of the duration of memory retention (26). In this classification system memory can be divided into: sensory memory, short-term memory, and long-term memory. Sensory memory is the retention of impressions of sensory information after the original stimulus has ceased. It refers to items detected by sensory receptors which are retained temporarily in the sensory inventory. Sensory inventory has a large capacity for unprocessed information but is only able to hold accurate images of sensory information momentarily. Without rehearsal, sensory memory typically lasts less than 1 sec and no more than 2 sec. Visual sensory memory is commonly called iconic memory, while auditory sensory memory is referred to as echoic memory. Typically tests of iconic memory involve exposing subjects to a grid of three rows of four letters for a brief period. Participants must then recall as many letters as they can and also the row in which the letters were found.

Short-term memory is defined as the capacity to hold a small amount of information in an active, readily available state. Short-term memory can hold a small amount of information from a few seconds to a minute without rehearsal. Like sensory memory, short-term memory has a limited capacity, estimated at 3 to 9 elements. Items held in short-term memory include: recently processed sensory input, recently retrieved

long-term memories, and the results of recent mental processing. A special form of short-term memory, working memory, refers to the temporary storage and manipulation of information needed for language comprehension, learning, and reasoning (27). Working memory is unique in that it requires the simultaneous storage and processing of information. Working memory can be generally divided into three subcomponents: 1. central executive, an attentional-controlling system important for problem solving tasks; 2. visuospatial sketchpad, manipulates visual images; and 3. phonological loop, stores and rehearses speech-based information.

Long-term memory can store information for as little as a few days or as long as decades. Unlike sensory and short-term memories, long-term memory has an unlimited capacity. Short-term memory is supported by a temporary potentiation of neuronal connections, especially in the frontal and parietal lobes. Specific patterns of neuronal connections distinguish different memories. A stronger neuronal connection encoding a stimulus will result in a stronger associated memory. Through rehearsal and association this temporary potentiation can be processed into long-term memory. The proposed mechanism by which short-term memories move to long-term memory is via long-term potentiation (LTP), which results in physical changes in the structure of neurons. Long-term potentiation is the long lasting enhancement in communication between two neurons simultaneously stimulated (28, 29). LTP shares several properties with long-term memory, making it a candidate for the cellular mechanism of learning. Both LTP and long-term memory are rapidly induced, stimulus specific, rely on the formation of new proteins, and last for months following exposure to the original stimulus (30). It is widely accepted that during long-term memory formation changes in protein synthesis

controls synaptic strength via rapid protein production from preexisting mRNA. Protein synthesis is required for several forms of synaptic plasticity (31-34). Synaptic plasticity is a physiological phenomenon whereby specific patterns of neural activity give rise to changes in synaptic efficacy and neural excitability that outlast the initial stimulus (35). It is thought that changes in synaptic patterns underlie the storage and recall of memory. Various neurotransmitters in the brain, including acetylcholine, γ-aminobutyric acid (GABA), adrenergic and glutamertergic systems have been shown to play a significant role in memory function (33-37).

Memory formation and storage involves complex communications between different cortical and subcortical structures of the brain, with no single site of memory formation and storage (38). The cerebellum, the limbic system (hippocampus, amygdala and the thalamus) and the cortical areas of the brain have been associated with memory storage and retrieval (39). Each structure has been associated with the encoding and retrieval of different memory types. Clinical case in humans (40-43) and animal studies of hippocampal lesions (43-46) demonstrate the importance of the hippocampus in memory acquisition. Specifically, it is thought that the hippocampus is important in short-term storage of sensory stimuli. Following the initial stimulus, the hippocampus coordinates the spontaneous retrieval of different components of an experience and relays these unique components to different memory systems depending on the memory type (47). Declarative memory is an aspect of human memory pertaining to the storage of facts, dates, and life events. This type of information is relayed to perirhinal cortex and medial temporal lobe (48-50). Memories of skills, procedural memory, is relayed to the striatum, neocortex, or to the cerebellum depending on the aspect of the skill. The

cerebellum is involved in the storage of reflexes while the striatum and neocortex stores information about the skills.

Two memory types studied during cognitive testing in rodents include working memory and reference memory. Working memory is defined as short term memory for an object, stimulus, or spatial location that is used within a testing session, but not typically between sessions. This is distinguishable from a form of long-term memory termed reference memory, which is a memory that is typically acquired with repeated training, and would persist for days to months. Reference memory is often the memory for the rules of a particular memory paradigm; for example, that an operant lever press produces a reward, or that a hidden platform is found in the Morris water maze. Working memory is typically a delay-dependent representation of stimuli that is used to guide behavior within the task. Working memory functions on a particular trial of a task, but then must be forgotten or ignored on subsequent trials (27).

Two major learning paradigms are commonly used in rodent studies of cognition. Conditional learning paradigms are used due to the ease of controlling the conditioned stimulus. Spatial learning tasks are among the most commonly used tests for detecting potential cognitive enhancers and cognitive changes associated with aging.

Conditioned learning tasks can involve aversive or reinforcement stimuli. Avoidance tasks measure memory of an aversive event, through simple avoidance of the location in which the aversive stimulus was first experienced. The animals avoid a location, (for example a dark room) where they previously received an aversive stimulus (foot shock). An avoidance task can require either a passive or active response. With a passive avoidance task an animal is placed in a lighted room; having a natural disposition

for the dark the rodent will leave the lighted room and enter the darkened room. Once it enters the dark room a foot shock is administered, providing a stimulus which helps the animal learn on subsequent trials to stay in the lighted room. When the aversive stimulus is made predictable the animal can then actively avoid the stimulus. In an avoidance paradigm, task performance is measured by the ability of the animal to avoid the compartment where a shock has occurred during the training phase of the paradigm. In reinforcement paradigms, animals can learn an operant lever press to gain access to an appetitive stimulus such as water or food.

In spatial learning tasks, the animal's behavior can be driven either by an aversive stimulus where the goal is to find refuge (finding a platform in the Morris water maze task), by an appetitive stimulus (water or food), or by spontaneous choice exploration (novel arm versus familiar arm). The Morris water maze requires rats to find the location of a submerged invisible platform in a large circular pool of water. One version of the task that tests reference memory is typically run by submerging the platform in the same location in the pool across days. The rat solves the task by learning the spatial relationships between the platform location and extramaze landmarks in the testing environment. Evidence of the rat learning the location of the hidden platform is observed with a shorter time latency to swim to the platform during subsequent trials. Working memory versions of this task have also been developed, with the location of the platform changed each day of testing. Working memory versions of the water maze require the animal to remember the location of the hidden platform during multiple trials in a training day, but forget this location on the next day of training. Again, evidence of the rat

learning the location of the hidden platform is seen with shorter latency to swim to the platform as the number of trials increase for that training day.

A classical task for testing memory in rodents is the radial arm maze. The maze is comprised of a center platform with radiating arms (6-12 arms). In this task, the rat is placed in the center platform, and a food reward is available at the ends of each of the arms. Working memory is tested by determining the number of arms entered before all of the food reward is recovered. It is observed that rats retrieve the food from each arm, and quickly learn to visit all the arms without re-entering a previously visited arm. A version of the radial arm maze has also been developed to test working and reference memory at the same time. In this version of the task, a predetermined number of arms are baited. The same maze arms are baited each day, across sessions, the rats must learn to ignore the unbaited arms. Entry into an unbaited arm is considered a reference memory error, while within a session re-entering a baited arm is considered a working memory error.

The T-maze task can be utilized to test delayed alternation or matching to position tasks. Delayed alternation tasks rely on the rats' tendency to choose alternative maze arms or locations when rats are re-exposed to the apparatus. The task works as follows: a rat is placed into the base (start location) of the T. The rodent runs up the stem of the maze and enters one arm of the T, where it then obtains a reward. The rat is picked up by the observer and returned to the base of the T. Typically the rat will run up the stem and enter the arm of the T that was not entered on the previous trial. Rats tend to alternate without reinforcement; which is referred to as spontaneous alternation.

A delayed matching to position task (DMP) can be used to offset the natural tendency of spontaneous alternation. This task is performed in a similar way to the alternation task except on the second trial the animal must enter the same arm as previously entered in order to receive the food reward. The DMP task typically requires more days of testing for the rodents to learn the task. The animal must remember the initial response to repeat the response, thus this is a working memory task. Typically, it is assumed that the rats solve the T-maze by remembering the location of the most recently visited arm based on its spatial relationship with extramaze cues. Remembering the spatial relationship of cues to guide behavior is considered allocentric spatial memory. The rat, however, may solve the task utilizing their directional sense, for example, first going east and then going west. The rat may also remember which turn it has made (left) and make the opposite or same turn on the subsequent trial. By doing this, the rat would adopt an egocentric strategy. Egocentric strategy, also term response learning, involves rotating in a self centered spatial framework in response to a cue. Guidance strategy consists of approaching or avoiding an object or cue in the environment. The use of this type of strategy is known as "cue learning". Finally, if subtle odor cues are left in the maze, the rat may detect the arm that was most recently entered by olfaction. It is unlikely that animals use only one learning strategy during acquisition of a T-maze task. It is more likely that learning in this task requires the adoption of multiple learning strategies during different periods of training.

Neurons in the hippocampus code spatial location (41-42), and performance within the T-maze is particularly sensitive to the effects of hippocampal disruption (37-40). Damage to the hippocampus and its connections produce significant impairments in

many forms of the T-maze task. Therefore, it is likely that damage to the hippocampus diminishes the ability of rodents to use spatial relationships in the environment to guide navigational behavior. These animals could still use either egocentric or guidance strategies, which are mediated by non-hippocampal structures.

3. Overview of Acetylcholine as a Neurotransmitter:

Acetylcholine (ACh) is a major neurotransmitter in both the mammalian peripheral nervous system and mammalian central nervous system (CNS) (51). Within the peripheral nervous system, acetylcholine is involved in the coordination of skeletal and smooth muscle contraction, control of heart rate, and regulation of endocrine gland secretions. Within the central nervous system, acetylcholine is implicated in emotional and attentional processes, learning, memory, and arousal (51-55).

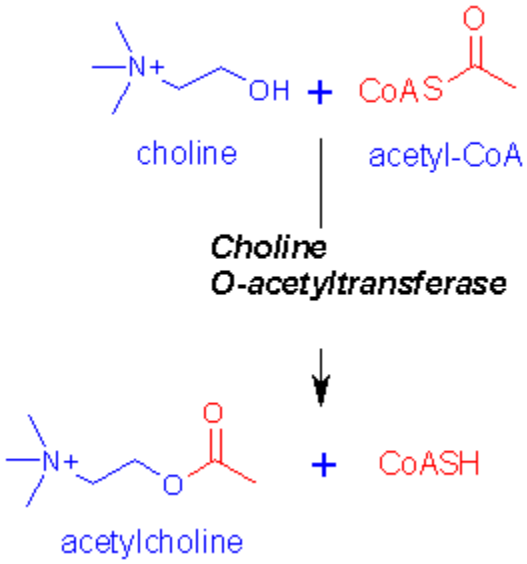


Illustration 1: The synthetic pathway for acetylcholine.

Acetylcholine is synthesized in a reaction catalyzed by the enzyme choline acetyltransferase (ChAT) from acetyl coenzyme A (CoA) and choline. Acetyl CoA primarily arises from glucose in the mitochondria through glycolysis and the pyruvate dehydrogenase system; from citrate, either by reversal of citrate synthesis or by citrate cleavage; or from acetate through acetate thiokinase (56,57). Following hydrolysis of ACh, 30-50% of the liberated choline is transported back into the presynaptic neuron through a high affinity choline transporter. The remainder of the choline is catabolized or incorporated into phospholipids where it can be used as a future source of choline. The uptake of choline is the rate limiting factor in the synthesis of ACh (56). Acetylcholine esterase normally hydrolysizes acetylcholine into its inactive metabolites. This enzyme is abundant in the synaptic cleft of neural tissue, and its role of clearing ACh from the synaptic cleft is essential for proper functioning of the cholinergic system (2, 56, 57).

Cholinergic receptors fall into two classes, muscarinic and nicotinic. Currently five muscarinic receptors (M_1-M_5) have been identified, each produced by a different gene. All muscarinic receptor subtypes are G-protein coupled metabotropic receptors that contain seven hydrophilic transmembrane domains, and transduce a signal that activates a number of second messenger systems. The M_1, M_3, M_5 receptors are coupled via Gq to phosphatidylinositol hydrolysis and M_2 and M_4 receptors via Gi are coupled to cyclic adenosine monophosphate. The cellular effects of muscarinic receptor activation include either opening or closing of K^+ channels, Ca^{2+} channels, or Cl^- channels, depending on the receptor and cell type. Therefore, the stimulation of muscarinic receptors can result in either depolarization or hyperpolarization of a neuron.

The nicotinic ACh receptor is a pentameric integral membrane protein composed of five different polypeptide chains (α, β, γ, δ, ε). To date 17 nicotinic ACh receptors have been identified, which are divided into muscle-type and neuronal-type subunits. Neuronal-type nicotinic receptors can be divided into two subtypes: 1.) the high affinity agonist binding class, which fails to bind α-bungerotoxin and is a heteropentameric nicotinic receptor formed by α2-α6 and β1-β4 subunits; and 2.) lower affinity binding class that binds α-bungerotoxin and is usually homopentameric formed by α7-α9 subunits (51). Acetylcholine binds to the α subunit causing a conformational change in the channel that selectively allows cations (Na^+ and Ca^+) to pass through the membrane, leading to a rapid depolarization and excitation. Nicotinic receptors are located at skeletal neuromuscular junctions, autonomic ganglia, adrenal medulla and CNS (56,57).

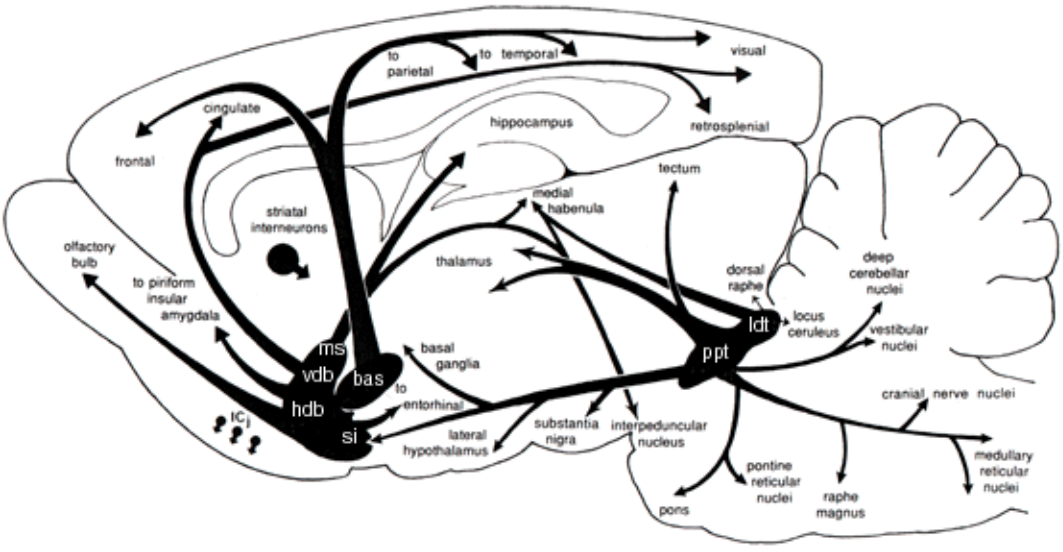


Illustration 2: Cholinergic system of the rat brain. Notice the major cholinergic innervation of the hippocampus by the medial septum. ms- medial septum, vdb- vertical diagonal band of Broca, hbd- horizontal diagonal band of Broca, bas- nucleus basalis magnocellularis, si- substantia innominate , ppt- pendunculopotine, ldt- laterodorsal tegmental nuclei (51).

The cholinergic system in the brain can be divided into four main components: the basal forebrain cholinergic system, pontine (brainstem) cholinergic system, motor neurons and cholinergic interneurons. The basal forebrain and pontine cholinergic systems contain projection neurons connecting two or more different regions. Cholinergic interneurons are classified as local circuit cells, which are morphologically arranged within one neuronal structure, and are exemplified by the interneurons of the caudate putamen nucleus, nucleus accumbens, olfactory tubercle and islands of Calleja complex. Motor neurons are located within the CNS and project their axons to the periphery where they directly or indirectly control muscle movements. Within the CNS, the motor neurons originate from the primary motor cortex, brain stem and cranial nerve nuclei. The pontine cholinergic system contains cholinergic neurons in the pendunculopotine and laterodorsal tegmental nuclei, which project to the thalamus, pontine, other diencephalic loci, medullary formations and cranial nerves (58).

Acetylcholine containing neurons of the basal forebrain located in the nucleus basalis magnocellularis (NBM), the diagonal band of Broca (DBB) and the medial septum (MS), give rise to projection neurons innervating the cerebral cortex, the amygdala, the hippocampus and several thalamic nuclei (59-60). The NBM contains the primary concentration of cholinergic neurons that project to the neocortex, while the DBB contains a cholinergic bundle of nerve fibers that interconnects the paraterminal gyrus, in the septal area, with the hippocampus and lateral olfactory area. The septohippocampal tract is formed by the cholinergic projection neurons from the MS to

the hippocampus and provides the greatest cholinergic innervation of the hippocampus (59,60).

It was observed that hippocampal ACh release increased during performance of a learned spatial memory task (61,62), and the improvement in radial arm maze performance was positively correlated to the increase in ACh release during 12 days of task learning (63). These results demonstrated that the learning of the spatial task modified the function of cholinergic neurons projecting to the hippocampus, which become progressively more active. Furthermore, spatial discrimination learning selectively increased muscarinic ACh receptor immunoreactivity in CA1-CA2 regions of the hippocampus, and in the neocortex, but not in the amygdala (64).

Memory processes are mediated by independent and/or competing parallel neuronal systems (for a review see 65). It has been demonstrated that hippocampal ACh release increases both when rats are tested in a hippocampal dependent spontaneous alternation task and an amygdala dependent conditioned place preference (CPP) task (66). The magnitude of hippocampal ACh release was negatively correlated with performance in the CPP task, indicating not only a competition between the two structures, but also that activation of the cholinergic hippocampal system inhibited the expression of amygdala dependent memory. However, competition was not the only interaction between the hippocampus and the amygdala, because it has been reported that ACh release in the amygdala was positively correlated with performance in a hippocampal spatial working memory task (66). The two structures seemed to have a nonreciprocal interaction in that the hippocampus competes with the amygdala, but the amygdala cooperates with the hippocampus during learning.

In the plus maze task, the time course of ACh release in the hippocampus and the striatum differed during training. The hippocampal cholinergic system was activated first during training and was involved in a place strategy depending on spatial memory; the striatal cholinergic system was activated later in training, when the rats shifted to response strategy to solve the maze (67). Studies indicated that the cholinergic projection neurons may not only be important for learning but may also be important for attentional processes (68). Microdialysis experiments indicated that cortical ACh increase during performance of a simple operant task was limited to early acquisition stages, when the attentional demand is high (69). Similarly, large increases in cortical and hippocampal ACh release were observed during acquisition of a reward operant task, but not during recall of the task (70). Intraparenchymal injections of a selective neurotoxin disrupted attentional processing in NBM- and MS-infused animals, thus confirming the role of the cholinergic system in attention (71).

4. Central Cholinergic Immunolesioning with SAP:

The cholinergic hypothesis of AD proposes that degeneration of cholinergic neurons in the basal forebrain and the associated loss of cholinergic neurotransmission in the cerebral cortex and hippocampus contribute significantly to the deterioration of cognitive function in AD patients (72). To study the contributions of cholinergic basal forebrain neurons to the establishment of the cognitive features of AD, animal models are required that specifically mimic cholinergic cell loss in the basal forebrain and cortical cholinergic hypoactivity. Classical methods that relied on fimbrial transactions, mechanical lesions, chemical injections of quisqualic acid, α-amino-3hydroxy-5-methyl-

isoxazole-4-proprionic acid (AMPA) and ibotenic acid failed to induce complete and selective cholinergic cell death in the basal forebrain (72-74). The main reason for this complication was that cholinergic neurons of the basal forebrain are intermingled with GABAergic and other neurons, which have similar projection targets that also influence memory function. Therefore, nonselective lesions confound the interpretation of the behavioral results from these animal models.

An understanding of the role of basal forebrain in learning and memory has accelerated since the development of immunotoxins such as 192-IgG-saporin (SAP). SAP provides a more efficient and selective tool to induce permanent cortical hypofunction. The immunotoxin was constructed through a disulfide bond linkage of the monoclonal antibody 192IgG and the plant alkaloid saporin (75). 192IgG is a monoclonal antibody raised against the low affinity nerve growth factor receptor (p75 NGF receptor), while saporin, obtained from Saponaria officinalis, is a potent ribosome-inactivator (76). p75 NGF receptors are extensively expressed on the soma and terminals of the cholinergic neurons of the basal forebrain (77). This neurotoxic complex binds to the p75 NGF receptor found on cholinergic neurons of the basal forebrain. Like the endogenous ligands for the p75 NGF receptor, the toxin is internalized after binding, and is reterogradely transported back to the soma (78). The saporin toxin is cleaved from the antibody and escapes the endosome, allowing saporin to enzymatically inactivate the large ribosomal subunit of the protein complex. Inactivation of this complex inhibits protein synthesis, ultimately leading to neuronal death (76).

Following ventricular injections, the immunotoxin is taken up by cholinergic neurons of the basal forebrain resulting in cholinergic losses in the MS, DBB, and NBM;

whereas, adjacent cholinergic neurons in the ventral pallidum and caudate putamen are spared (79). The MS and DBB contain cholinergic and GABAergic neurons which both project to the hippocampus. Ventricular injections had no effects on the GABAergic neurons of the basal forebrain, but affected purkinje fibers which also express p75 NGF receptors (79). The latter needs to be considered during behavioral testing since destruction of the purkinje fibers have been associated with severe motor deficits in rats.

Histological experiments demonstrated that localized infusion of SAP into the three distinct loci of the basal forebrain resulted in a complete loss of NGF immunoreactive neurons within the areas of interest (80). Following localized infusions of SAP, sections stained for a cholinergic marker, AChE, also showed a loss of cholinergic neurons in distinct loci. A dramatic reduction of AChE staining in the target areas (dorsolateral neocortex, cingulated cortex and hippocampus) was associated with the distinct region of the basal forebrain which provides cholinergic innervation. Similarly ChAT activity in the targeted areas displayed reduced activity, further demonstrating cholinergic hypoactivity (81). Localized infusion of SAP did not affect cholinergic neurons which lacked p75 NGF receptor expression. Localized infusions of higher SAP doses (1 μg) directly into the basal forebrain induced lesions of all cholinergic neurons within the basal forebrain and other non-cholinergic neurons expressing p75 NGF receptors including the purkinje fibers (82). Smaller doses of SAP (0.22 μg) injected directly into selected loci of the basal forebrain, like the MS, spared not only the purkinje fibers but also GABAergic neurons present in the MS (82). Effects on GABAergic neurons should be considered because of the important role they play in regulating hippocampal function. The intensity of staining for parvalbumin, a calcium

binding protein expressed by a subset of GABAergic neurons in the MS, is examined to detect changes in the population of GABAergic neurons present in the MS. At the 0.22 μg dose of SAP, there is no effect observed in the intensity of parvalbumin staining, compared to the decreased staining with higher doses of SAP (82). Thus, injections of low doses of SAP directly into individual basal forebrain loci could provide an excellent tool for dissociating the role of cholinergic basal forebrain nuclei and their projections in mediating cognitive processes.

5. Role of Dehydroepiandrosterone and Dehydroepiandrosterone Sulfatase in Cognition:

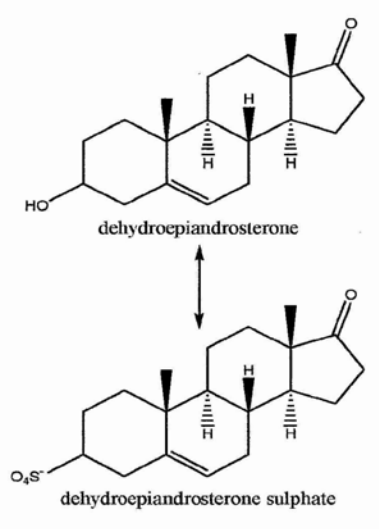


Illustration 2: Structural relationship between dehydroepiandrosterone and its sulfated form

Dehydroepiandrosterone (DHEA) and its sulfated ester dehydroepiandrosterone sulfate (DHEAS) have gained significant interest in the field of neuroscience due to two findings: a strong age associated decline of the steroid in humans and the demonstration of DHEA(S) metabolism and action in the rodent brain. DHEAS is the most abundant

steroid product of the adult human adrenal cortex with concentrations in the microgram per milliliter range (83). In addition to its adrenal origins, DHEA is also produced in small amounts (10-20% of circulating hormone) within the gonads and CNS (84,85). Formation of DHEA is stimulated by adrenocorticotropic hormone from the pituitary, has a short serum half-life (30 min. or less), and a serum concentrations that fluctuate markedly with the circadian rhythm. DHEAS has serum concentrations greater than 200 times that of DHEA and has a much longer half-life (7-10 hours), which largely removes the variation in plasma concentration associated with circadian rhythm (86-90). Due to the short half life (30 min) and quick catabolism of DHEA, less than 1% of the steroid is in circulation as the unsulfated form (91). Most of the DHEAS (60-80%) entering the blood is converted to DHEA by steroid sulfatase, while a small amount of DHEA (5-7%) is converted to DHEAS by hydroxysteroid sulfotransferase in the liver, kidney, and CNS, creating an equilibrium between the two isoforms (89, 92). DHEA(S) exhibits a strong and unique developmental pattern.

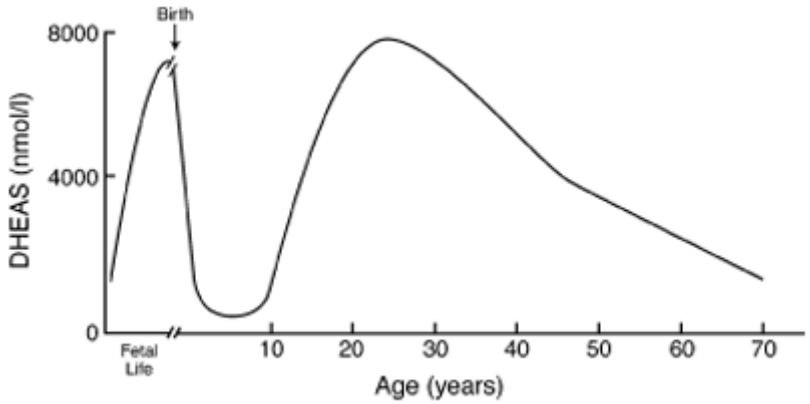


Illustration 3: Unique developmental pattern for DHEAS

DHEA is produced in the fetal adrenal glands, with concentrations sharply dropping and becoming almost undetectable 6 months following birth (93). One to two years after puberty a rise in DHEA production occurs, with a peak concentration obtained during the third decade of life (83, 94-95). Thereafter, a marked continuous decline in DHEA(S) concentration is observed with patients in their sixth decade of life, possessing only 20% of plasma concentrations compared to pubescent patients. This developmental pattern is observed in both males and females, with males having higher DHEAS levels in adulthood (96-99). This strong age-associated pattern is not observed for cortisol, the primary human glucocorticoid secreted from the adrenal cortex, where basal levels are unchanged or slowly increase throughout the life-span (100, 101).

Neuroactive steroids are endogenous compounds with neuromodulatory effects (102). Several of these compounds, such as estrogens and corticosterone, have been shown to enhance cognitive function in rodents and have gained significant interest as potential therapeutic regiments for dementia and age-associated memory loss (for a review see 103). In the rodent brain, estrogen supplementation has been shown to enhance ChAT activity, high affinity choline uptake, and the release of ACh in the hippocampus following potassium stimulation (104-106). Moreover, studies have demonstrated that estrogen supplementation can reduce the incidence and severity of Alzheimer's disease in post menopausal women (107).

A subset of neuroactive steroids, termed neurosteroids, accumulates in the nervous system independent of the products of steroidogenic endocrine glands. Neurosteroids are synthesized *de novo* in the nervous system from sterol precursors. Neurosteroids have also been shown to modulate neuronal activity within the central

nervous system (102). There is substantial evidence that DHEA and DHEAS are produced within the CNS and are therefore neurosteroids (102). DHEA(S) concentrations in the rodent brain far exceed peripheral concentrations and are independent of adrenal synthesis (108, 109). However, the exact metabolic pathway(s) for DHEA(S) in the rodent brain are still not completely understood, since P 450-17α (17 α hydroxylase; the enzyme which converts pregnenolone into DHEA) is present in the fetal, but not in the adult rodent brain (110). Furthermore, within the rodent brain there is a small amount of hydroxysteroid sulfotransferase activity. These findings suggest that DHEA, which passes the blood brain barrier more readily than DHEAS, could be locally converted to DHEAS in the brain (111, 112). Sulfatase activity is widely present in the CNS and its pharmacological inhibition affects memory (113-117). Others have suggested an alternative pathway for the synthesis of DHEA in the central nervous system involving sterol and/or steroid hydroperoxides. One study in primates reported high DHEAS levels in the brain that were not strongly suppressed by dexamethasone treatment, suggesting local biosynthesis (118). Moreover, a *postmortem* study in human tissues demonstrated higher levels of DHEA and DHEAS in brain tissue compared to blood concentration, again suggesting local biosynthesis (119).

Flood *et al.* were the first to show the memory-enhancing effects of DHEA and DHEAS, in both young (120-122) and old (123) mice using a foot shock paradigm. In their extensive studies, multiple routes of administration (i.c.v., s.c., oral) were utilized, all leading to an inverted U-shaped dose response curve for memory-enhancing effects. Melchior and Ritzmann (124) demonstrated that when administered i.p., both DHEA and DHEAS enhanced short-term working memory as assessed with a T-maze paradigm.

Again, an inverted U-shaped dose response curve was observed. Another group of experiments demonstrated that DHEAS enhanced memory when given before or directly after training, but not when administered before retention; suggesting that DHEAS enhanced the storage and/or consolidation of the learned paradigm but not retrieval (125).

Other studies have investigated the anti-amnestic properties of DHEA(S) using a variety of amnestic agents. Flood *et al.* were again the first to show the anti-amnestic properties of excitatory neurosteroids using multiple routes of administration (120-122). Maurice *et al.* confirmed the antiamnestic effect of DHEA and DHEAS in mice utilizing multiple memory paradigms (Y maze, water maze, passive avoidance) with multiple amnestic agents (126-128). Moreover, Maurice *et al.* demonstrated that DHEAS had effects on learning and/or storing information but no effect on recall of the same information (127).

DHEA(S) has memory enhancing and anti-amnestic effects on several brain locations associated with memory function, suggesting a global effect rather than actions limited to particular structures. Paradigms utilized to show the memory enhancing effects of DHEA(S) rely on different neuronal structures, especially the hippocampus, the amygdala and frontal cortical regions (for review see 129).

Only one study reported memory impairment after DHEAS treatment (130). DHEAS administered orally for 5 days impaired hippocampal-dependent contextual fear conditioning, while having no effect on auditory cue fear conditioning. Following adrenalectomy, similar effects were reported, providing evidence for an extraglucocorticoid action of DHEA. The results of this experiment could be explained by a DHEAS-induced potentiation of the corticosterone response to a stressful test

paradigm, thus shifting the optimal corticosterone response, thereby impairing hippocampal plasticity. This interpretation is supported by the observations that the identical DHEAS treatment enhanced memory performance in a T-maze memory paradigm (a less stressful task of hippocampal-mediated memory in rodents); thereby demonstrating that the memory impairing effects of DHEAS are only observed if the hippocampal meditated memory is tested under stressful conditions (131).

Since DHEA(S) concentrations decrease significantly with age in humans and multiple beneficial effects of DHEAS have been documented in rodents, many studies have investigated the relationship between DHEA(S) and cognition in humans. High-functioning elderly patients, as defined by cognitive and functional examinations, possess higher DHEAS levels compared to patients in the median or lower functioning groups (132, 133). Lower DHEAS levels were also associated with poorer health, impaired global functioning, and physiological well being (134). However, several short term studies found no beneficial effects of DHEA on well-being or cognitive performance in old or young adults (135, 136). Recent studies have shown that among Alzheimer's disease patients, those with higher concentrations of plasma DHEAS performed better on tests of association, digit span, and mini mental status exams when compared to those with lower levels of this neurosteroid (137).

The exact mechanisms by which DHEA(S) enhance memory function in the rodent remain unclear. It appears that DHEA(S) acts primarily through interactions with cell surface neurotransmitter receptors. This is in contrast to the classical actions of steroids that include: binding to an intracellular receptor, translocation to the nucleus, and binding to a response element resulting in altered protein synthesis. The best documented

effects of DHEA(S) within the CNS are via negative modulation of the $GABA_A$ receptor complex (138-141). This ligand-gated ion channel is a multimeric transmembrane receptor consisting of five subunits arranged around a central pore, which upon activation, increases chloride influx into the cell. Imamua *et al.,* demonstrated that DHEA and DHEAS noncompetitively inhibited GABA induced currents in cultured rat neurons with DHEAS being 3-4 times more potent than DHEA (142). Moreover it was suggested that DHEAS may interact with the barbiturate sites on the $GABA_A$ receptor. Others have suggested picrotoxin as the DHEAS binding domain with DHEA having no effects on GABA currents (143). There are several studies in rodents that show that $GABA_A$ antagonists enhance memory (144, 145) and their agonists impair memory (146, 147). These same effects are also relevant to humans, with benzodiazepines impairing cognition.

In addition to modulating $GABA_A$ currents, DHEAS also interacts with sigma receptors. The sigma receptor (σ_1 and σ_2) is expressed in many tissue types, but is particularly concentrated in the CNS (148). Many antipsychotic and antidepressant drugs have high binding affinity for the receptor (149). Selective sigma agonists potentiate the response of rat CA3 dorsal hippocampal neurons to N-methyl-D-aspartate (NMDA) which suggests a functional link between the two receptor types. The ability of sigma receptors to modulate NMDA mediated glutamatergic neurotransmission has been emphasized and proposed to play an important role in major neuro-adaptative events, such as long-term potentiation, learning and memory, and neurodegeneration (149-152). The first *in vitro* study for the role of DHEAS in modulating sigma receptors demonstrated that DHEAS (30mM or higher) potentiated NMDA evoked release of

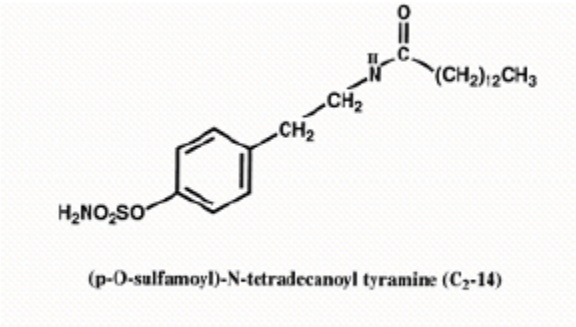
norepinephrine from preloaded hippocampal slices (153). Pertussis toxin, which inactivates G_i protein function, suppressed this effect of DHEAS (153). Since σ_1 but not σ_2 receptors are coupled to G_i proteins, these results suggest that DHEAS modulates the NMDA response via the σ_1 receptor. These conclusions are further supported by a recent *in vivo* binding study in mice (154). Sigma receptor agonists enhanced memory performance in several paradigms in young as well as in a model for cognitive aging in rodents (153, 154). NMDA receptor involvement in memory is suggested by its important role in the development of long-term potentiation and the finding that NMDA receptor antagonists produce amnesia in both humans and rodents (143).

There are several possible pathways by which neurosteroids enhance memory function. The GABAergic neurons of the nucleus accumbens are known to synapse upon cholinergic neurons of the MS which then form the major cholinergic projections to the hippocampus (155). Therefore, DHEA(S) may enhance memory by disinhibiting the cholinergic neurons of the MS and increasing concentrations of acetylcholine in the hippocampus. In support of this hypothetical pathway, peripheral administration of DHEAS enhanced hippocampal ACh release in vivo, and this enhancement occurred in a dose-dependent manner (155). In further support of this hypothesis, Yoo *et al.* demonstrated a dose dependent increase in hippocampal long term potentiation following treatment with DHEAS (156).

6. Effects of Steroid Sulfatase Inhibitors on Learning and Memory

There is now good evidence that the metabolism of DHEA and its sulfated form occurs bi-directionally within the CNS, with DHEAS being desulfated via the enzyme

steroid sulfatase (108). (p-O-sulfamoyl)-N-tetradeconoyl tyramine (DU-14) is a potent non-estrogenic irreversible inhibitor of steroid sulfatase. A single dose of DU-14 (30 mg/kg) was able to inhibit 95.2% of the steroid sulfatase activity within the liver (116). Furthermore, this treatment was able to significantly lower steroid sulfatase activity within the brain but at a substantially lower rate (14.8% reduction in activity) (116). Chronic treatments with DU-14 (30 mg/kg for 15 days i.p.) in rodents increased plasma concentrations of DHEAS, while decreasing plasma DHEA (113). This same treatment also increased whole brain levels of DHEAS.



(p-O-sulfamoyl)-N-tetradecanoyl tyramine (C_2-14)

Illustration 4: Chemical structure of (P-o-sulfamoyl)-N-tetradeconoyl tyramine

The administration of DU-14 had been shown to increase hippocampal ACh release, a result which supports the findings that increased DHEAS levels in the brain augment the levels of ACh within the hippocampus (157). Additionally, chronic pretreatment with DU-14 attenuated scopolamine induced spatial memory impairment in the passive avoidance paradigm and Morris water maze (116, 158). In the Morris water maze not only did DU-14 reverse the scopolamine induced amnesia, but enhanced the performance of control animals (116). In agreement with the GABA antagonistic action

of DHEAS, these findings suggested that DHEAS rather than DHEA was responsible for memory enhancement. Since all of these experiments administered the steroid sulfatase inhibitor through an i.p. route, it remains unclear whether the observed effects were caused by actions of the inhibitor at the peripheral or central level. It was hypothesized that DU-14 could also attenuate the cognitive deficit associated with lesion of septal-hippocampal cholinergic projections.

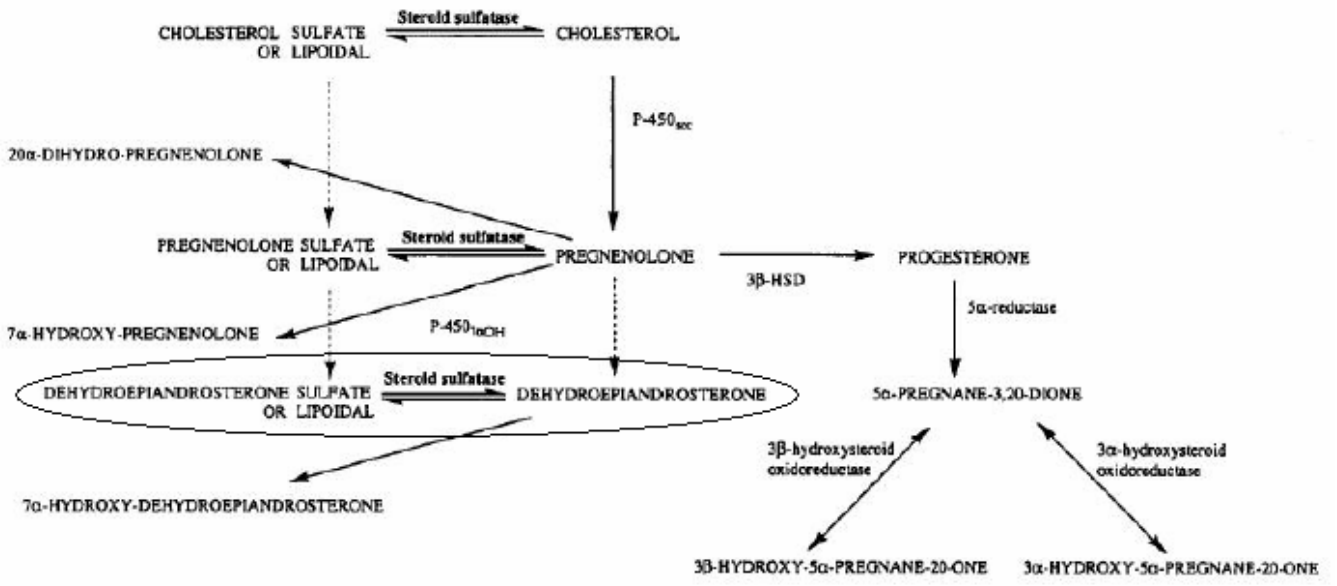


Illustration 5: Neurosteroid biosynthesis and metabolism in the rat brain. Dotted arrows indicate metabolic conversion not yet formally demonstrated. Note: figure is a modification of Figure 1 found in (226)

7. Effects of Aversive Stimulus and Stress on Spatial Learning

Emotional, aversive, and stressful experiences, via the activation of specific hormonal and brain systems, alter learning and memory processes (159-161). Stress is a factor that is often overlooked when conducting research with laboratory animals. Stress

exposure, depending on intensity and duration, can elicit either positive or negative effects on cognitive functions, particularly spatial learning and memory performance (162). Stress-induced responses are largely mediated by the hypothalamic-pituitary-adrenal (HPA) axis and result in increased plasma concentrations of both adreno-corticotropic hormone and corticosterone, released from the anterior pituitary and adrenal cortex, respectively. Short periods of mild stress (163-164) enhanced acquisition of certain spatial learning tasks, while longer periods of stress, or corticosterone treatment, impaired performance on a variety of spatial tasks (165-166).

The ability of stress to enhance performance of a spatial task may be due to the activation of pathways independent of the septal-hippocampal tract. The glucocorticoids secreted in response to stress notably bind to mineralcorticoid and glucocorticoid receptors in the hippocampus. Furthermore, epinephrine released from the adrenal cortex as a consequence of mild stressors that occur during a memory task can activate adrenergic receptors that facilitate memory function (167-169). Adrenergic receptors found on vagal afferents project to the nucleus of the solitary tract and can subsequently activate neurons that project to the amygdala (164). The amygdala has long been associated with the acquisition of memories related to aversive stimuli (170-174) and the modulation of memory processes of the hippocampus (175-177).

Based on these data, it is hypothesized that the mechanisms and pathways that underlie stress-induced enhancement of task performance, including spatial memory tasks, are distinct from the septal cholinergic neurons. It is also hypothesized that the insertion of an aversive stimulus into a non-aversive DMP task could attenuate the deficit in acquisition previously observed from the lesion of septal cholinergic neurons. This

could explain the lack of consistency in the literature regarding the importance of MS cholinergic neurons in spatial working memory. Several studies have shown that selective destruction of the cholinergic neurons of the MS induced a marked decrease in acquisition of a spatial task (82, 105, 178). Other investigations, though, have reported only limited effects of SAP lesions in the MS on spatial tasks. For example, Dornan *et al.*, (179) reported that a selective reduction of cholinergic transmission in the basal forebrain was by itself insufficient to account for the functional impairments in spatial learning of rats using a Morris water maze paradigm. Baxter *et al.* (180, 181) utilizing a Morris water maze reported similar findings. Therefore, a task with a mildly aversive component may preserve acquisition of a spatial task in animals with a septal-hippocampal lesion via pathways that are independent of the septal-hippocampal tract. The result would be a loss of sensitivity in paradigms which involve a mildly aversive stimulus when assessing impairments in learning/memory associated with septal-hippocampal lesion.

B. Statement of the Problem

Alzheimer's disease, the most common form of dementia, is a progressive neurodegenerative disorder characterized by memory loss and behavioral changes. Decreases in acetylcholine levels in multiple memory systems like the hippocampus and cortical regions of the brain have been implicated in the pathogenesis of Alzheimer's disease. Currently, animal models used to study the effects of cholinergic hypofunction on learning and memory formation are limited. Animal models must be developed which can mimic the cholinergic hypofunction seen early in the pathology of many forms of dementia. Once these models are developed, detailed studies are needed to determine the importance of cholinergic projections to the hippocampus and other cortical regions for learning and memory processes.

The current therapies of choice for dementia are anticholinesterase agents, which increase synaptic acetylcholine levels by inhibiting the enzyme acetylcholinesterase. However, these treatments provide only symptomatic relief and are ineffective in treating the disease itself. DHEAS, a neurosteroid, replacement has been shown to be effective in reversing memory deficits associated with scopolamine induced amnesia. Furthermore, preventing the conversion of these neurosteroids, especially DHEAS, to the unsulfated form can enhance memory function. No study has thus far investigated the effect of DHEAS treatment on impairments in acquisition of a spatial task due to loss of septal-hippocampal cholinergic neurons. Furthermore, it has yet to be determined whether preventing the conversion of these neurosteroids to the unsulfated form through chronic administration of a steroid sulfatase inhibitor can attenuate the cognitive deficits associated with the selective loss of septal-hippocampal cholinergic projections.

C. Hypothesis and Aims

1. Hypothesis

A selective loss of cholinergic neurons projecting to the hippocampus from the medial septum results in diminished acquisition of a delayed matching-to-position T-maze paradigm. DHEAS, through direct administration or prevention of its conversion to an unsulfated form, will improve acquisition of the delayed matching-to-position T-maze paradigm independent of cholinergic projections to the hippocampus.

2. Specific aims

1. Examine the changes in the acquisition of a delayed matching-to-position T-maze paradigm following selective cholinergic immunolesion of the medial septum with 192 IgG-saporin.

2. Examine changes in the adoption of different learning strategies in a spatial T-maze task following selective cholinergic immunolesion of the medial septum with 192 IgG-saporin.

3. Examine the ability of DU-14 to attenuate cognitive deficits produced by the destruction of cholinergic neurons projecting to the hippocampus utilizing delayed matching-to-position T-maze paradigm.

4. Examine the ability of DHEAS to attenuate cognitive deficits produced by the destruction of cholinergic neurons projecting to the hippocampus utilizing delayed matching-to-position T-maze paradigm.

5. Examine the effects the introduction of an aversive stimulus into the appetitive T-maze paradigm has on the acquisition in rats with a selective 192 IgG-saporin lesion of the medial septum.

II. MATERIALS AND EQUIPMENT

1. Facilities

Laboratories – Richard King Mellon Hall of Science, Room 416a
Bayer Learning Center, Animal Facility

Office – Richard King Mellon Hall of Science, Room 420

2. Laboratory Animals

300 – 350 gram Male Sprague-Dawley Rats
Hilltop Lab Animals Inc., Scottdale, PA

3. Chemicals and Drugs

192 IgG-saporin
Chemicon, Temecula, CA

Artificial Cerebral Spinal Fluid
CMA Inc., North Chelmsford, MA

Veterinary Sterile Saline
Henry Schein, Melville, NY

Veterinary Sterile Water
Henry Schein, Melville, NY

Pentobarbital

Triadine Solution
Stoelting Co., Wood Dale, IL

Surgilube Surgical Lubricant
Stoelting Co., Wood Dale, IL

Isopropyl Alcohol
Eckerd, Pittsburgh, PA

Ethanol
Fischer Scientific, Pittsburgh, PA

Mazola Corn Oil
Giant Eagle, Pittsburgh, PA

Acetaminophen
Sigma Chemical Co., Saint Louis, MO

Neosporin Antibiotic Ointment
Lab Safety Supply Inc., Janesville, WI

Dehydroepiandrosterone sulfate
Sigma Chemical Co., Saint Louis, MO

(P-o-sulfamoyl)-N-tetradeconoyl tyramine (DU-14)
Gift from Pui-Kai Li at Ohio State University

Saline Tablets
Sigma Chemical Co., Saint Louis, MO

Ethylenediaminetetraacetic acid (EDTA)
Sigma Chemical Co., Saint Louis, MO

Triton X-100
Sigma Chemical Co., Saint Louis, MO

^{3}[H] acetyl-CoA
Sigma Chemical Co., Saint Louis, MO

Choline chloride
Sigma Chemical Co., Saint Louis, MO

Physostigmine sulfate
Sigma Chemical Co., Saint Louis, MO

Sodium Chloride
Sigma Chemical Co., Saint Louis, MO

Sodium phosphate buffer
Sigma Chemical Co., Saint Louis, MO

Acetonitrile
Sigma Chemical Co., Saint Louis, MO

Tetrephenylboron
Sigma Chemical Co., Saint Louis, MO

EconoFluor scintillation cocktail
Packard Instruments, Meriden, CT

4. Materials

Hamilton 7000 5.0 μl Microsyringe
Stoelting Co., Wood Dale, IL

Tungsten Micro Drill
Stoelting Co., Wood Dale, IL

Nylon Monofilament Sutures
Stoelting Co., Wood Dale, IL

Original Perry Style Sterile Surgical Gloves
Stoelting Co., Wood Dale, IL

Surgical Mask
Stoelting Co., Wood Dale, IL

Bouffant Caps
Stoelting Co., Wood Dale, IL

Shoe Cover
Stoelting Co., Wood Dale, IL

NU Gaze
Stoelting Co., Wood Dale, IL

Sterile Cotton Tipped Applicators
Stoelting Co., Wood Dale, IL

Disposable Sterile Surgical Drape
Stoelting Co., Wood Dale, IL

Sterilizing Pouches
Stoelting Co., Wood Dale, IL

Dissecting Forceps
Stoelting Co., Wood Dale, IL

Micro-Dissecting Retractors
Stoelting Co., Wood Dale, IL

Micro Dissecting Scissors
Stoelting Co., Wood Dale, IL

Bone Ronguers
Stoelting Co., Wood Dale, IL

Needle Holder
Stoelting Co., Wood Dale, IL

Scalpel Blades (#10)
Stoelting Co., Wood Dale, IL

Scalpel Handlers
Stoelting Co., Wood Dale, IL

Pipetman Pipettes
Fisher Scientific, Pittsburgh, PA

BD Falcon Conical Tubes (50, 15 ml)
Fisher Scientific, Pittsburgh, PA

Eppendorf tubes (0.6, 1.5 ml)
Fisher Scientific, Pittsburgh, PA

BioRad Protein Assay
BioRad, Hercules, CA

Scintallation Vials
Fisher Scientific, Pittsburgh, PA

Fisherbrand Redi-Tips disposable pipette tips (200, 1000µl)
Fisher Scientific, Pittsburgh, PA

BD syringe (1.5, 10ml)
Fisher Scientific, Pittsburgh, PA

Disinfecting Cleaner
Lab Safety Supply Inc., Janesville, WI

FEP Tubing
CMA Inc., North Chelmsford, MA

Infusion Probe
CMA Inc., North Chelmsford, MA

Reward Pellets
Research Diets, New Brunswick, NJ

LabDiet Rodent Chow
Purina Mills, St. Louis, MO

5. Equipment

Accumet pH Meter, Model 291
Fisher Scientific, Pittsburgh, PA

Themolyne Nuova Stirrer
Fisher Scientific, Pittsburgh, PA

Fisher Scientific Accu-124 Scale
Fisher Scientific, Pittsburgh, PA

Revco Freezer (-80°C)
Revco Thermo Electron Corporation, Asheville, NC

Whirlpool Refrigerator
Whirlpool Corp., Benton Harbor, MI

Autoclave

Lab Standard Stereotaxic Instrument
Stoelting Co., Wood Dale, IL

Infusion Syringe Pump
Stoelting Co., Wood Dale, IL

Germinator Dry Sterilization
Stoelting Co., Wood Dale, IL

Rodent Brain Matrices (large coronal rat)
Stoelting Co., Wood Dale, IL

Decapitator
Stoelting Co., Wood Dale, IL

Homeothermic Blankek for Rodents
Stoelting Co., Wood Dale, IL

Surgical Clippers
Stoelting Co., Wood Dale, IL

Rotating T-Maze
Consists of: Approach alley: 4” wide x 14” long
Two Goal Arms: 4” wide x 12” long
Black acrylic walls 5” high
Clear acrylic hinged top
Mounted to a “Lazy Susan”

Sonicator
Fisher Scientific, Pittsburgh, PA

Liquid Scintillation Counter
Packard Instruments, Meriden, CT

UV/VIS Spectrophotometer
Beckman Instruments, Fullerton, CA

6. Computer Software

Graphpad Prism 3.0
Graphpad, San Diego, CA

Microsoft Excel, Microsoft Office XP
Microsoft Corporation, Orem, UT

Microsoft Word, Microsoft Office XP
Microsoft Corporation, Orem, UT

Microsoft Photoshop, Microsoft Office XP
Microsoft Corporation, Orem, UT

Microsoft PowerPoint, Microsoft Office XP
Microsoft Corporation, Orem, UT

Adobe Photoshop Version 5.0
Adobe Sytems, Seattle, WA

Adobe Acrobat (Reader and Writer)
Adobe Sytems, Seattle, WA

III. EXPERIMENTS

A. Effects of medial septum cholinergic lesions on ChAT activity.

1. Introduction

SAP is a powerful immunotoxin that has been shown to selectively destroy the p75 NGF receptor expressing neurons that are extensively expressed on the soma and terminals of the cholinergic neurons of the basal forebrain (75,77). Histological experiments demonstrated that localized infusion of SAP into the three distinct loci of the basal forebrain resulted in a complete loss of NGF immunoreactive neurons within the area of interest (80). Following localized infusions of SAP, sections stained for a cholinergic marker, AChE, showed a loss of cholinergic neurons in the distinct loci. Furthermore, there was a dramatic reduction of AChE staining in the target areas (dorsolateral neocortex, cingulated cortex, and hippocampus) associated with these distinct loci of the basal forebrain. Similarly, ChAT activity in the targeted areas displayed reduced activity, further demonstrating decreased cholinergic innervation (81). Smaller doses of SAP (0.22 μg) injected directly into selected loci of the basal forebrain, like the MS, spared not only the purkinje fibers but also GABAergic neurons present in the MS (82). Therefore, we hypothesized that low doses of SAP can be used to selectively destroy the cholinergic neurons of the MS. We evaluated whether infusions of SAP (0.22 μg) into the MS significantly lowered the ChAT activity in the hippocampus, while displaying no effect on frontal cortex ChAT activity. These results will validate the selective loss of cholinergic neurons present in the MS and the sparing of the cholinergic neurons in the NBM following the SAP infusion. ChAT activity in the hippocampus and frontal cortex was also used to determine correct placement of the

cannula during the SAP lesioning process. We predicted that small dose of SAP (0.22 μg) infused directly into the MS will result in complete loss of cholinergic innervation of the hippocampus from the MS. Infusions of small doses of SAP can then be used as a model to explore the importance of the MS cholinergic innnervation of the hippocampus in different learning and memory tasks. SAP treated animals that did not show selective loss of ChAT activity in the hippocampus were excluded from subsequent studies.

2. Methods

i. 192 IgG-saporin Immunolesioning:

All experiments followed the guidelines of the N.I.H. for the care and use of laboratory animals and were approved by the Duquesne University Institutional Animal Care and Use Committee. Male Sprague-Dawley rats weighing between 300 and 350 grams were obtained from Hilltop Lab Animals (Scottdale, PA). Male rodents were chosen so that influences of estrogens on learning and memory were minimized. Animals were housed individually in a temperature and humidity controlled facility on a 12:12 h light:dark cycle with unrestricted access to food and water. For intraseptal infusion, animals were anesthetized with pentobarbital (50mg/kg; IP) and placed into a stereotaxic frame. An incision was made exposing the dorsal aspect of the skull and a hole was drilled over the medial septum (MS; +0.2 bregma, 0.0 lateral). A stainless steel cannula was lowered 5.6mm from the dura into the MS. Animals were infused with either 1 μl vehicle (artificial cerebral spinal fluid [aCSF]) or SAP (0.22μg in 1 μl of aCSF) over 5 min. at a rate of 0.2μl/min. The dose of SAP was selected based on previous studies using the same lot number (Advanced Targeting Systems; Lot #24-87)

that demonstrated a substantial loss of cholinergic neurons in the MS with little nonselective damage to GABAergic neurons (82). Following infusion, the cannula remained in place for 5 min. to allow for diffusion of the solution into the tissue, and prevent the removal of SAP from the intended injection site. The incision was sutured closed and animals were allowed to recover for 2 weeks before initiating behavioral testing.

ii. Choline Acetyltransferase Assay (ChAT):

The degree of cholinergic deafferentation was assessed by measuring ChAT activity within the hippocampus and frontal cortex. After completing all training, animals were anesthetized with pentobarbital (100mg/kg; IP) and perfused with ice cold saline. The brains were removed, and tissues from the hippocampus and frontal cortex dissected, frozen, and stored at -80°C until processed for ChAT activity as previously described (82). Frozen tissues were thawed and dissociated by sonication in a medium containing EDTA (10mM) and Triton X-100 (0.5%) and diluted to a concentration of 10mg tissue/ml. An aliquot of each sample was used for the determination of total protein (186). Three 5µl aliquots of each sample were incubated for 30 min. at 37°C in a medium containing ^{3}[H] acetyl-CoA (50,000-60,000d.p.m./tube, final concentration 0.25mM acetyl-CoA), choline chloride (10.0mM), physostigmine sulfate (0.2mM), NaCl (300mM), sodium phosphate buffer (pH 7.4, 50mM), and EDTA (10mM). The reaction was terminated with 4 ml sodium phosphate buffer (10mM) followed by the addition of 1.6mL of acetonitrile containing 5mg/ml tetraphenylboron. The amount of ^{3}[H] acetylcholine produced was determined by adding 8 ml of EconoFluor scintillation

cocktail and counting total cpms in the organic phase using an LKB beta-counter. Background was determined using identical tubes to which no sample was added. The three reaction tubes containing sample were averaged and the difference between total cpms and background cpms was used to estimate the total amount of ACh produced per sample. Cholineactyltransferase activity was then calculated for each sample as pmol Ach synthesized/hr/μg protein.

iii. Statistical Analysis:

Due to the large number of samples, ChAT assays were completed with multiple runs, with each run containing samples from different treatment groups and normalized to percent change from controls. Differences in ChAT activity for the hippocampi of treatment groups were determined using one-way ANOVA. All analyses were performed using GraphPad Prism 3.0.

3. Results

Levels of ChAT activity are summarized in Table 1a and 1b. As expected, infusion of SAP (0.22μg/μl) into the medial septum (MS) resulted in a significant decrease in ChAT activity within the hippocampus, demonstrating a loss of cholinergic afferents from the MS to the hippocampus. There was a 70.5% decrease in ChAT activity in the hippocampi of SAP-MS treated rats compared to control animals. (7.85 ± 1.37 versus 26.61 ± 0.77 pMol ACh/h/μg protein; $p < 0.001$). There was no significant difference in ChAT activity between treatment groups for tissues collected from the frontal cortex ($p = 0.26$).

Table 1a: ChAT activity in aCSF-treated control animal

	Range	Means ± s.e.m.
Hippocampus (n=45)	25.03 – 31.10	26.61 ± 0.77
Frontal Cortex (n=45)	27.21 – 40.92	34.12 ± 3.06

Values expressed as pMol ACh produced/h/μg protein.

Table 1b: ChAT activity in SAP-MS treated animal

	Range	Means ± s.e.m.
Hippocampus (n=42)	3.83 – 13.34	7.85 ± 1.37
Frontal Cortex (n=42)	22.24 – 44.83	30.01 ± 3.62

Values expressed as pMol ACh produced/h/μg protein.

4. Discussion

The ChAT assay results demonstrated that infusion of SAP (0.22μg/μl) into the MS resulted in a significant decrease of ChAT activity within the hippocampus, but not in the frontal cortex (Table 1a-1b). This finding is consistent with previous studies that showed a substantial loss of cholinergic neurons in the medial septum, but not in the nucleus basalis magnocellularis (NBM) using the same infusion protocol (82, 187, 188). The selectivity of SAP for cholinergic neurons in the MS has been questioned. High doses of SAP injected into the lateral ventricles led to the loss of non-cholinergic p75NTR-expressing neurons located in distal from the infusion site (197). Many of these distal effects were avoided by infusing small amounts of SAP directly into the MS. Since GABAergic innervation of the hippocampus plays an important role in regulating hippocampal function it is important to select a dose of SAP that will not affect other neuron types at the site of infusion. Parvalbumin is a calcium binding protein that serves as a marker for GABAergic neurons. Previous studies have shown that the dose (0.22μg/μl) from the lot of SAP used in the present study had little effect on GABAergic parvalbumin-containing neurons located in the same regions of the MS (82). Therefore,

it is concluded that any learning deficits observed in SAP treated groups are the consequence of a selective septal-hippocampal cholinergic lesion. SAP treated animals that did not display selective destruction of the MS cholinergic neurons were eliminated from all subsequent experiments.

B. Effects of medial septum cholinergic lesions on spatial performance.

1. Introduction

To study the contributions of cholinergic basal forebrain neurons to the establishment of the cognitive features of AD, animal models are required that specifically mimic cholinergic cell loss in the basal forebrain and cortical cholinergic hypoactivity. This experiment was designed to better understand the role of the cholinergic projections from the MS during learning and memory. Several studies have shown that selective destruction of the cholinergic projections from the MS induced a marked decrease in acquisition of a spatial task (82, 105, 178). However, other investigations have reported only limited effects of SAP lesions in the MS on spatial tasks. For example, Dornan *et al.*, (179) reported that a selective reduction of cholinergic transmission in the basal forebrain was by itself insufficient to account for the functional impairments in spatial learning of rats using a Morris water maze paradigm. Baxter *et al.* (180, 181) utilizing a Morris water maze reported similar findings. It is possible that other tests may be more sensitive for detecting cognitive deficits associated with the selective loss of cholinergic projections from the MS, particularly tests which assess learning as opposed to working memory and retention. We hypothesized that a selective loss of cholinergic neurons projecting to the hippocampus from the MS results in diminished acquisition of a DMP T-maze paradigm. We predict that SAP treated animals

will take significantly more days to reach criterion and acquire the DMP task at a significantly slower rate. If learning and memory deficits are observed following SAP lesioning of the MS, this could serve as a model to test drug therapies targeted at attenuating these memory deficits. Furthermore, this can be used as a model to determine the importance of the MS cholinergic neurons in AD pathology.

2. Methods

Immunolesioning of the cholinergic neurons of the MS with SAP was performed as documented in Experiment A. All animals were allowed to recover 2 weeks following surgery before initiation of the behavioral testing. Following behavioral testing ChAT activity in the hippocampus and frontal cortex was determined for each animal as described in Experiment A. SAP treated animals that did not show selective decrease in ChAT activity within the hippocampus were eliminated from the study.

i. Dietary Restriction-

Following surgery animals remained on an *ad libitum* diet. The day before the first session of T-maze acclimation, all food was removed from the housing cages. Diet restrictions began by feeding the animals 4 pieces of Rodent Chow (~8 g) after completion of the T-maze acclimation. Weight was monitored daily, adjusting the amount of chow so that the animal maintained ~85% of normal body weight throughout the study. A control animal fed *ad libitum* was included with each experimental group to ensure that the weights of the experimental animals did not exceed 85% of a normal age matched controls. Animals were fed after completion of T-maze activities.

ii. Behavioral testing:

The delayed match-to-position (DMP) task was performed as recently described (82, 182, 183). All behavioral testing was performed between 10:00 a.m. and 2:00 p.m., seven days a week, except following the introduction of the probe trial. During the probe trial a morning session (9:00 a.m. to 12 p.m.) and afternoon session were both performed (2:00 p.m. to 5 p.m.). The order of testing for the different groups of animals was changed daily. DHEAS displays minimal variation in plasma concentration associated with circadian rhythm, while DHEA has a short serum half-life and serum concentrations that fluctuate markedly with the circadian rhythm (86-90). Varying the order by which the animals were tested minimizes the impact that variations due to circadian rhythms of DHEA and other neuroactive steroid levels has on learning and memory.

T-maze Acclimation:

Acclimation began by scattering ten reward pellets throughout the T-maze with all doors of the T-maze opened. Randomly paired animals were placed into the T-maze for 10 minutes. Before running the next pair of animals, the T-maze was cleaned with a non-toxic spray disinfectant.

On the second day of acclimation, ten reward pellets were scattered throughout the T-maze and all doors of the T-maze remained open. Sweetened rodent chow (50 mg, sucrose sweetened chow) was used throughout all experiments as the reward pellet. Animals were placed individually into the T-maze for 5 minutes. Again, the T-maze was cleaned with a non-toxic spray disinfectant following each acclimation session.

On days three through five of acclimation, four reward pellets were placed in each goal arm of the T-maze (total of 8 pellets) and all doors of the T-maze remained open. Animals were placed individually into the T-maze for 5 minutes or until all 8 pellets were eaten.

Animals were then trained to run to the ends of each goal arm by using a series of 6 forced choices on days six through eight of acclimation. Animals were placed individually into the start box of the T-maze with the approach alley door and one goal arm door closed. After 10 seconds of confinement in the start box, the approach alley door was opened, allowing the animal to explore the maze. Once the animal entered the open goal arm, 2 reward pellets were dropped into this arm. The animal was allowed to explore the T-maze for 90 seconds or until both reward pellets were eaten. After each trial pair, animals were returned to their housing cages for 5-10 minutes, while training proceeded on the remaining animals of the group. To avoid the introduction of a side bias, right and left goal arms were alternated in a random and balanced fashion according to the below list.

DAY 6	**DAY 7**	**DAY 8**
Left	Right	Left
Right	Left	Left
Left	Right	Right
Right	Left	Right
Left	Right	Left
Right	Left	Right

Delayed Match-to-Position Task Testing:

The DMP task was chosen to offset the natural tendency for spontaneous alternation observed in rodents (184, 185). Animals were placed individually into the start box of the T-maze with the approach alley door and one goal arm door closed. After 10 seconds, the approach alley door was opened allowing the animal to move out of the start box and down the alley. Once the animal entered the open goal arm, 2 reward pellets were placed in the arm. The animal was allowed to explore the T-maze for 1 minute or until both reward pellets were eaten. The animal was then immediately returned to the start box and the maze was cleaned with nontoxic disinfectant spray to remove any odor cues. Both goal arm doors were opened followed by the approach alley door. The rodent was allowed to explore the T-maze for 1 minute or until it entered a goal arm. If the animal entered the same physical goal arm (correct choice) that it explored during forced choice, 4 reward pellets were placed in the arm. After the animal had eaten the pellets, it was returned to its housing cage for 5 to 10 minutes while training on the remaining animals in the group was finished. If the animal entered the opposite goal arm (incorrect choice) as sampled during the forced choice, the goal arm door was closed and the animal was confined to that goal arm for 30 seconds. After the 30 seconds of confinement, the animal was returned to its housing cage for 5 to 10 minutes while training on the remaining animals in the group was finished. Animals continue to receive eight trials per day until a criterion of 15 correct choices out of 16 consecutive trial pairs were met.

The following is the pattern in which all animals were run during DMP acquisition.

DAY 1	**DAY 2**	**DAY 3**
Left	Right	Right
Left	Left	Left
Right	Right	Right
Right	Left	Left
Left	Right	Right
Left	Left	Left
Right	Right	Left
Right	Left	Right

DAY 4	**DAY 5**	**DAY 6**
Right	Left	Right
Right	Right	Left
Left	Left	Left
Left	Left	Right
Right	Right	Left
Right	Left	Right
Left	Right	Left
Left	Right	Right

DAY 7	**DAY 8**	**DAY 9**
Left	Left	Right
Right	Left	Left
Right	Right	Right
Left	Right	Left
Right	Left	Right
Left	Right	Right
Left	Left	Left
Right	Right	Left

DAY 10	**DAY 11**	**DAY 12**
Right	Left	Right
Left	Right	Right
Right	Right	Left
Left	Left	Left
Left	Right	Right
Right	Right	Left
Right	Left	Left
Left	Left	Right

DAY 13:	**DAY 14**:	**DAY 15**:
Right	Left	Left
Right	Left	Right
Left	Right	Right
Right	Left	Left
Left	Right	Left
Left	Left	Right
Right	Right	Left
Left	Right	Right

DAY 16:	**DAY 17**:	**DAY 18**:
Left	Right	Left
Right	Left	Left
Left	Right	Right
Right	Left	Right
Right	Left	Left
Left	Right	Right
Left	Left	Right
Right	Right	Left

DAY 19:	**DAY 20**:	**DAY 21**:
Left	Left	Right
Right	Left	Right
Right	Right	Left
Left	Left	Right
Left	Right	Left
Right	Right	Right
Right	Left	Left
Left	Right	Left

DAY 22:	**DAY 23**:	**DAY 24**:
Left	Right	Right
Right	Left	Left
Left	Left	Right
Right	Right	Right
Right	Left	Left
Left	Right	Right
Right	Right	Left
Left	Left	Left

DAY 25:	**DAY 26**:	**DAY 27**:
Right	Left	Right
Left	Right	Left
Left	Left	Right
Right	Left	Right
Right	Right	Left
Left	Right	Left
Right	Left	Right
Left	Right	Left

DAY 28:	**DAY 29**:	**DAY 30**:
Left	Right	Right
Right	Left	Left
Left	Right	Left
Right	Right	Right
Left	Left	Right
Right	Left	Left
Left	Right	Left
Right	Left	Right

iii. Statistical Analysis:

Days to criterion were analyzed using Student's T-test for individual comparisons of the two treatment groups. To compare treatment effects during different stages of acquisition, performance data was blocked into 3-day periods of training. After an animal had reached criterion, a value of 93.75% (15/16) was recorded for performance on subsequent days. Performance during acquisition testing was analyzed using two-way ANOVA for overall effects of block and treatment and Student's T-test for treatment effects within blocks. Both the rate of performance and days to reach criterion in the DMP t-maze task was determined to be parametric. Power analysis and variability seen in similar studies (82, 188) suggests that 8 animals per treatment group were enough to determine statistical significance. All analyses were performed using GraphPad Prism 3.0.

3. Results

Of the original 19 animals, all but two animals failed to reach criterion on the delayed matching to position (DMP) task or displayed a side bias and were excluded from the study. One of the two animals was an aCSF control animal while the other received SAP lesioning of the MS. Analysis of the remaining animals (8 control animals

and 9 SAP) indicated that SAP treated animals required more days to reach criterion (Fig. 1). Control animals took an average of 16.00 ± 1.195 days while the animals that received SAP lesioning of the MS took an average of 21.56 ± 3.779 to complete the DMP task. Mann-Whitney non-parametric U-test showed a significant effect on treatment (p = 0.001), indicating that SAP treated animals differed significantly from the aCSF treated control animals.

Examining of the learning curves (Fig. 2) showed that all animals performed at similar levels, below chance, at the start of DMP training and SAP treated animals improved at a slower rate when compared to aCSF-treated controls. Analysis of the learning curves by two-way ANOVA demonstrated a significant effect of "Treatment" ($F[1,490] = 64.10$, $p < 0.0001$), a significant effect of "Block" ($F[9,490] = 159.82$, $p < 0.0001$), and a significant 'Tretament' X 'Block' interaction ($F[9,490] = 7.86$, $p = 0.0001$). A separate analysis of performance within a block revealed aCSF-treated animals performed significantly better than SAP treated animals during blocks 4-7 of training.

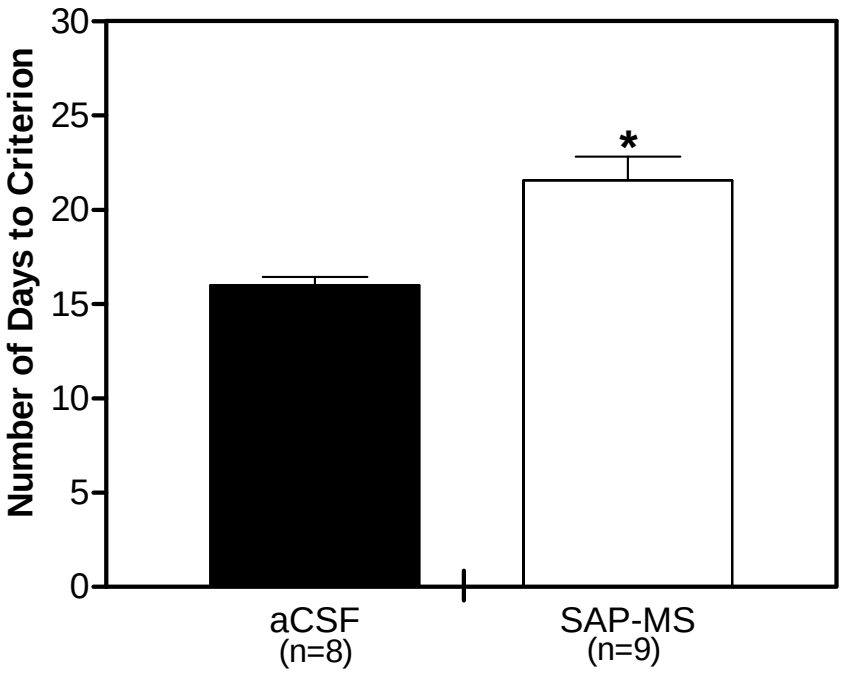


Figure 1. Effects of SAP-lesion of the MS on the number of days to reach criterion in the DMP T-maze task.

Bar graph of days to reach criterion in the DMP T-maze task. Top of bar represents the mean number of days to reach criterion ± standard error of the mean. Note that SAP-MS treated animals required significantly more days to reach criterion than the aCSF- treated controls. * $p <$ 0.05 relative to the aCSF-treated control animals.

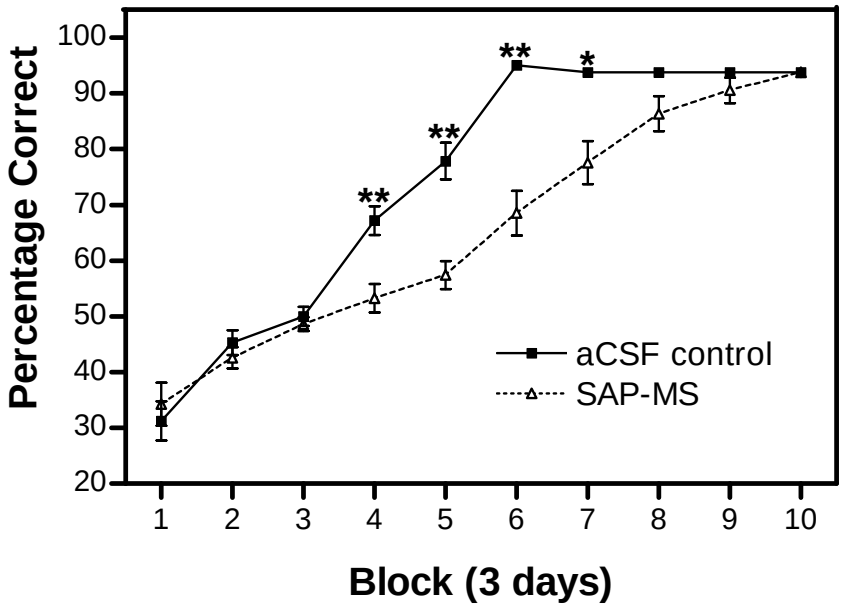


Figure 2. Effects of SAP-lesion of the MS on the rate of acquisition in the DMP T-maze task.

Learning curves show the effects of SAP lesioning of MS on the rate of DMP acquisition. Points represent the mean percentage correct for each treatment group during a 3 day period of training. By day 21 of training, all control animals had reached criterion, and only four of nine SAP treated animals had reached criterion. By day 30 all animals had reached criterion. Note that both groups showed improved rates of performance over time; however, during blocks 4–7 the rate of performance of the SAP treated animals was significantly impaired compared to the control animals. ** $p < 0.0001$, * $p < 0.05$ for aCSF treated control animals relative to each of the SAP treated groups.

4. Discussion:

The behavioral results show that SAP treated animals were significantly impaired in the acquisition of the DMP task. Specifically SAP treated animals took significantly longer to reach criterion and improved at a slower rate compared to controls (Fig. 1, 2). The specific cognitive processes disrupted following SAP lesion of the MS still remain unknown.

The motor activity of the different treatment groups during the acclimation phase of the DMP T-maze task suggests that there is no change in the motor activity following the SAP infusion. Both treatment groups could readily transverse the maze and obtain the reward pellets. Furthermore, there was no difference in the ability of SAP treated animals to explore an open field as compared to aCSF treated controls. This suggests that the decrease in the rate of acquisition and the increase in days to reach criterion was not influenced by the motor activity of the different treatment groups.

Several studies have reported limited to no effects of SAP lesion of the basal forebrain on tests of spatial working memory in rats. Mild working memory deficits were reported using a delayed non-matching to sample radial arm maze following SAP lesion of the MS (189). No deficits in memory recall were reported in rats that acquired a spatial paradigm followed by SAP lesioning of the MS and retesting (190). Likewise, there was no reported effect of SAP lesion of the MS, NBM, or MS plus NBM on spatial working memory in the Morris water maze (179-181). Moreover, Cahill and Baxter (191) reported facilitated acquisition of a discrimination task following SAP-lesions to the MS, suggesting no deficit in spatial working memory. No significant performance differences were reported between SAP lesions of the MS or control rats when the

intertrial delay between the forced and open choice was increased in the DMP task once animals reached criterion (82,188). Together, these findings suggest that any spatial working memory deficit produced by selective loss of the septal-hippocampal cholinergic neurons is mild at best, and not likely to account for the diminished acquisition of the DMP task following SAP lesion of the MS.

Other reports suggest that cholinergic neurons of the basal forebrain are more important for attentional processes than working memory processes. For example, several studies have reported significant deficits on tests of visual attention following SAP lesions of the NBM (192-194). Cholinergic projections from the MS were less involved in visual attention processes (195), but may influence other attentional processes important in acquisition of a spatial task.

Rats have a natural tendency for spontaneous alternation, which affects performance on spatial alternation tasks (184, 185). In a simple alternation task, many rats performed better than chance on the first day of acquisition (184, 185). As a result, it is often difficult when using a simple alternation paradigm to assess the rate of acquisition and overall performance of the animals. It was reported that infusions of SAP into the NBM of rats had no effect on the acquisition of a T-maze alternation task (196). Examination of the learning curves showed that the performance of control animals was near criterion, and that SAP lesioned animals performed better than chance at the beginning of training. Employing a T-maze alternation task, therefore, would make any effect on learning difficult to assess. By utilizing the DMP paradigm, the natural tendency of rats to alternate is opposed, thereby increasing the initial difficulty of the task and the sensitivity of the task to detect changes in task acquisition. This was evident on

the first day of training, where both the control and SAP lesioned rats perform below chance (Fig. 2). Following day 1 of training, the performance of all animals improved with control animals quickly surpassing the rate of acquisition for SAP lesioned rats. The DMP paradigm provided a sensitive behavioral assay for revealing selective deficits in the septo-hippocampal cholinergic pathway, while the specific cognitive process underlying the decreased rate of acquisition remained unclear. One of the most popular theories for the role of the hippocampus states that a map of the environment is built up in the hippocampus and serves to guide the animal within its environment (197). According to this theory, animals with hippocampal hypofunction are unable to use spatial strategies (based on a cognitive map), but can use either an orientation or guidance strategy, which are mediated by non-hippocampal structures. A possible explanation for the decreased rate of acquisition could be a decrease ability to use a spatial strategy or greater reliance on another learning strategy.

C. Effects of medial septum cholinergic lesions on learning strategies.

1. Introduction

Recently, investigations utilizing the selective cholinergic immunotoxin 192-IgG Saporin (SAP) have found that lesioning cholinergic neurons of the MS has little or no effect on working memory in tests such as the Morris water maze (179, 180, 190). However, as documented in Experiment B this same lesion significantly delays acquisition of a DMP T-maze task. Rats can utilize a number of different strategies to solve spatial tasks (for a review, see (27)). For example, an animal may utilize a consistent directional body turn to navigate a maze using either egocentric or allocentric

cues, or a spatial strategy that relies on the location of extra maze visual cues (198-200). Evidence suggests that turning strategies rely primarily on striatal circuits whereas spatial strategies rely more on hippocampal circuits (198, 200-202). As an example, when the dorsal hippocampus was inactivated via an infusion of lidocaine, use of a place strategy was inhibited (199). Conversely, following stimulation of the dorsal hippocampus with glutamate, rodents preferentially chose a place learning strategy (199). Therefore, another mechanism by which MS cholinergic lesions could affect DMP acquisition independent of working memory is by affecting the use and/or preservation of a place or response strategy. The purpose of this study was to test the hypothesis that MS cholinergic lesions impair DMP acquisition in male rats by causing alterations in the adoption and/or preservation of the learning strategies used within the DMP task. We predicted that SAP treated animals will use a persistent turning strategy for significantly more days, influencing the number of days the animal needs to reach criterion in the DMP task and the rate of acquisition for the task.

2. Methods

Immunolesioning of the cholinergic neurons of the MS with SAP was performed as documented in Experiment A. Following behavioral testing ChAT activity in the hippocampus and frontal cortex was determined for each animal as described in Experiment A. SAP treated animals that did not show selective decrease in ChAT activity within the hippocampus were eliminated from the study.

i. Post-criterion Spatial Manipulation:

Fourteen days after surgery for SAP infusion, animals were food deprived to 85% of their normal body weight and then acclimated to the T-maze as described in Experiment B. Acquisition of the DMP task, described in Experiment B, continued until two stages of learning were established. One group of animals (criterion performance) received eight trial pairs per day until the animals acquired the DMP paradigm, defined as 15 correct choices out of 16 consecutive trial pairs, while another group (response like performance) received training until 15 out of 16 entries into the same goal arm was observed. Once either criterion was reached, spatial manipulation was introduced into the testing procedure. During a morning session animals were run as described for the DMP task. During the afternoon session, animals completed the forced choice, but before the second open choice of the trial pair, the maze was rotated 180º. The animals were then placed in the start box and allowed to complete the second part of the trial pair. Use of a response like strategy was defined as entering the same physical goal arm in at least 7 out of 8 trials per day. A place strategy was defined as returning to the goal arm located in the same location within the testing environment 7 out of 8 times.

ii. Statistical Analysis:

During the course of DMP training it was observed that throughout early stages of training, many animals adopt a turning strategy whereby they consistently turned to the right or left goal arm of the maze. To quantify this observation, we counted the total number of days the animals utilized the strategy during the testing process. Any animal that entered 7 out of 8 times into the same goal arm was defined as using a turning

strategy. A student T-test was used to analyze the number of animals that adopted this strategy and also the number of days that an animal utilized the strategy. To evaluate the contribution that the turning strategy had on the number of days to reach criterion, the duration of the turning strategy was subtracted from the overall days to criterion from each animal and analyzed with a student T-test. Effects of rotating the maze 180° between the open and forced chioce were analyzed by ANOVA comparing the performance during a normal morning session with performance in an afternoon session when the maze was rotated. Performance and days to criterion were analyzed as parametric data.

One goal of the research was to test the predictions about the relationship between cholinergic activity in different brain regions and the different learning strategies adopted by each treatment group. ChAT activity was used to measure the cholinergic activity with in the hippocampus and frontal cortex. To compare the number of days with a turning strategy and ChAT activity, a scatter plot was developed and analyzed utilizing a Spearman's Rho correlation between groups and within groups. All analyses were performed using GraphPad Prism 3.0.

3. Results

Animals appeared to adopt different learning strategies at different times during training of the DMP task. To determine changes in learning strategies, a probe trial was introduced once animals reached two different stages of learning. We observed that during the early stages it appeared that animals adopted a turning strategy, where animals entered the same arm during the open trial no matter which arm they entered in the forced

trial. Therefore, the criterion for the first stage of learning that was examined was defined as 15/16 turns into the same arm of the T-maze as during the open trial. Prior observations have shown that animals reach this criterion early in training (days 3-6). The second stage of learning was followed by additional training when an animal reached the DMP criterion, defined as 15/16 correct choices (days 16-24). After reaching either criterion, each rat received a probe trial over three consecutive days during which the maze was rotated 180° (relative to the extra-maze cues) between the forced and open trials. Animals using extra-maze cues would enter the arm in the same location of the room as previously visited. This would be the opposite arm visited during the forced trial, thus an incorrect choice. Animals relying on internal cues would be expected to enter the same physical arm of the T-maze entered during the forced trial no matter the orientation of the maze in the testing room: a correct choice.

A probe trial introduced during the earlier stage of learning had no significant effect on the performance (Fig. 3A–3B) of either aCSF-treated controls or SAP treated animals. During a morning session of normal DMP training, aCSF treated animals displayed an average performance of 47.40 ± 1.06% correct, compared to the probe trial when animals displayed an average performance of 50.00 ± 1.51% correct. SAP treated animals also displayed similar behavior with an average performance of 48.75 ± 0.86% correct during normal DMP training and an average performance of 49.38 ± 2.31% correct during the probe trial.

Since animals seemed to adopt a turning strategy at this early stage in training, the number of times an animal entered a single arm of the T-maze during the open trial was also examined. The probe trial introduced during this stage of learning had no significant

effect on the established turning strategy for either the aCSF treated or SAP treated animals (Fig. 4A–4B). During the morning session of normal DMP training, the aCSF treated animals averaged 7.83 ± 0.08 turns into a single goal arm of the T-maze during the open trial, compared to the probe trial when these same animals averaged 7.46 ± 0.19 turns into the same arm. SAP treated animals displayed similar behavior. During a normal training session SAP treated animals averaged 7.86 ± 0.14 turns into a single arm during the open trial, compared to the probe trial when these same animals averaged 7.71 ± 0.28 turns into the same arm. The performance and entry patterns suggest that during this stage of learning, rotating the maze had no effect on arm choice in each of the treatment groups. This result indicates that during the early stages of learning, neither treatment group used extra-maze cues to a significant degree to navigate the maze.

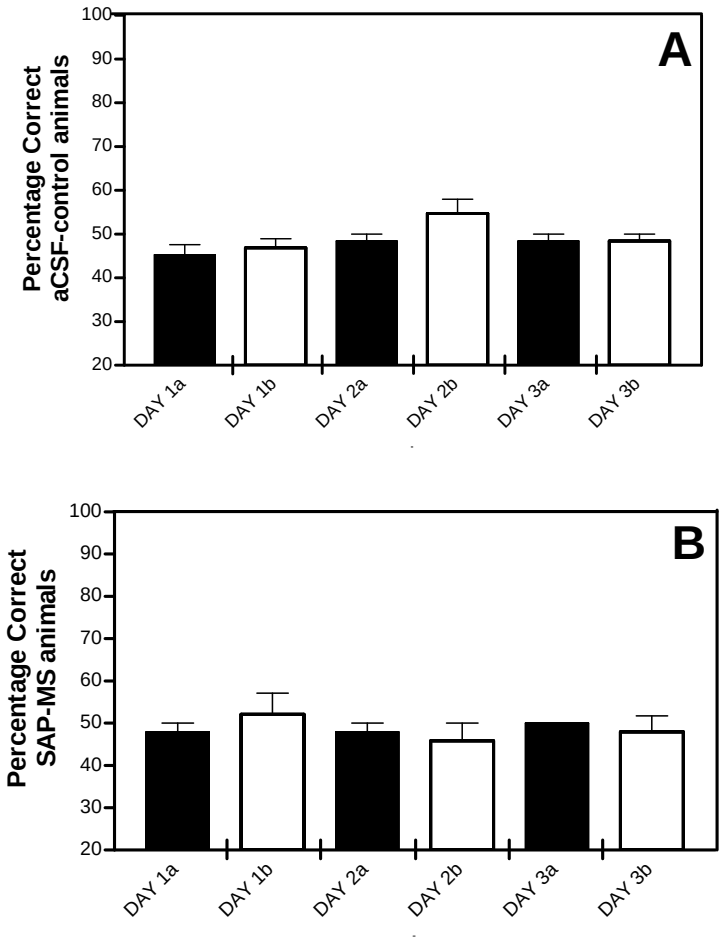


Figure 3A - 3B. Effects of rotating the T-maze on performance of animals using persistent turning strategy.

The performance of aCSF treated control animals (n=8) and SAP treated animals (n=6) during a normal trial pair (days 1a – 3a) and the probe trial (days 1b – 3b) over three consecutive days. Performance during the early stages of training when animals exhibited a turning strategy as defined by 15/16 turns to the same arm. The bars represent the mean percentage correct ± s.e.m. Note that introducing a probe trail had no significant effect on performance of either aCSF treated controls or SAP treated animals.

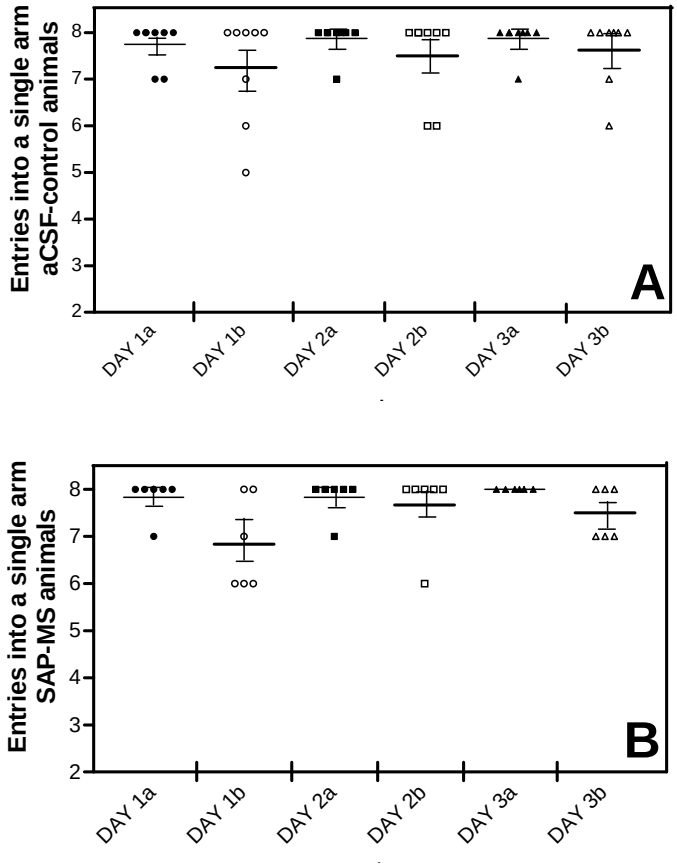


Figure 4A – 4B. Effects of rotating the T-maze on entry patterns of animals using a persistent turning strategy.

Scatter plots showing entry patterns for aCSF treated control animals (n=8) and SAP treated animals (n=6) during a normal trial pair (day1a-3a) and the probe trial (day1b-3b) over three consecutive days. Entry patterns summarized is during the early stages of training when animals exhibited a turning strategy defined by 15/16 turns to the same arm. The line in the middle represents the mean ± s.e.m. Note that introducing a probe trail had no significant effect on entries into a single arm of the T-maze for either aCSF treated controls or SAP treated animals.

A probe trial introduced following additional DMP training had a significant effect on the performance for both aCSF treated and SAP treated animals that had reached criterion in the DMP task (Fig. 5A–5B). During three consecutive days of the probe trial, the average performance was 53.13 ± 3.63% for aCSF treated controls and 63.02 ± 2.76% for SAP treated animals. These values differed significantly from performance during the normal training session (93.23 ± 2.13% for aCSF treated controls; 94.27 ± 1.50% for SAP treated animals; $p < 0.001$ for all days). Changes in performance indicated that following additional training, rotating the maze disrupted arm choice for both treatment groups. This result indicated that for the rats to solve the T-maze DMP paradigm they relied on extra-maze cues when making an arm choice.

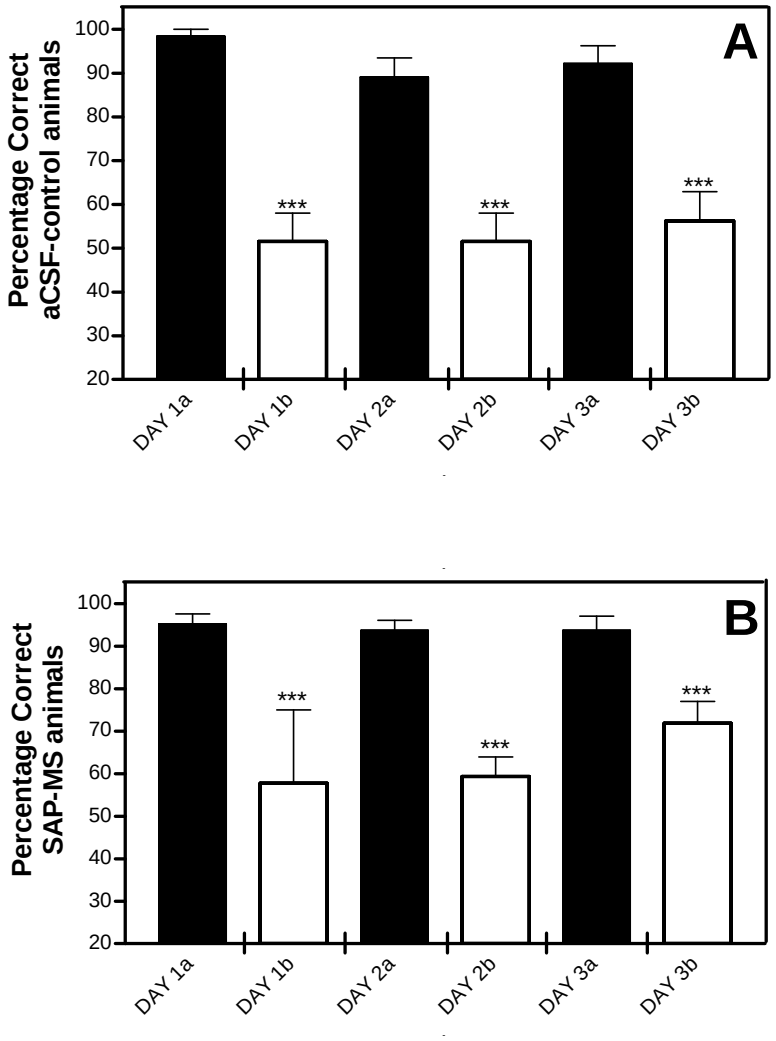


Figure 5A – 5B. Effects of rotating the T-maze on animals that have reached criterion following extensive training.

The performance of aCSF treated control animals (n=8) and SAP treated animals (n=8) during a normal trial pair (days 1a – 3a) and the probe trial (days 1b – 3b) over three consecutive days. Performance summarized is following extensive training when animals have solved the maze and chose 15/16 correct. The bars represent the mean percentage correct ± s.e.m. Note that introducing a probe trail significantly decreased the performance of both aCSF treated controls and SAP treated animals. *** $p < 0.001$ relative to the normal trial pair (days 1a – 3a).

As mentioned above, we observed that during early stages of training many of the animals adopted a turning strategy. Of the eight aCSF treated control animals, six developed a turning strategy (75%), defined as 15/16 turns to the same goal arm. All nine SAP treated animals adopted the turning strategy (100%). There was no significant difference in the number of days before each of the groups of animals adopted the turning strategy. aCSF treated controls took an average of 4.5 ± 1.6 days to adopt this learning strategy while SAP treated animals took an average of 4.7 ± 0.9 days. The number of days that animals engaged in the turning strategy was significantly greater for rats with SAP lesion of the MS (Fig. 6). SAP treated animals engaged in a turning strategy for 14.33 ± 1.81 days while aCSF treated controls engaged in this strategy for 6.63 ± 1.71 days.

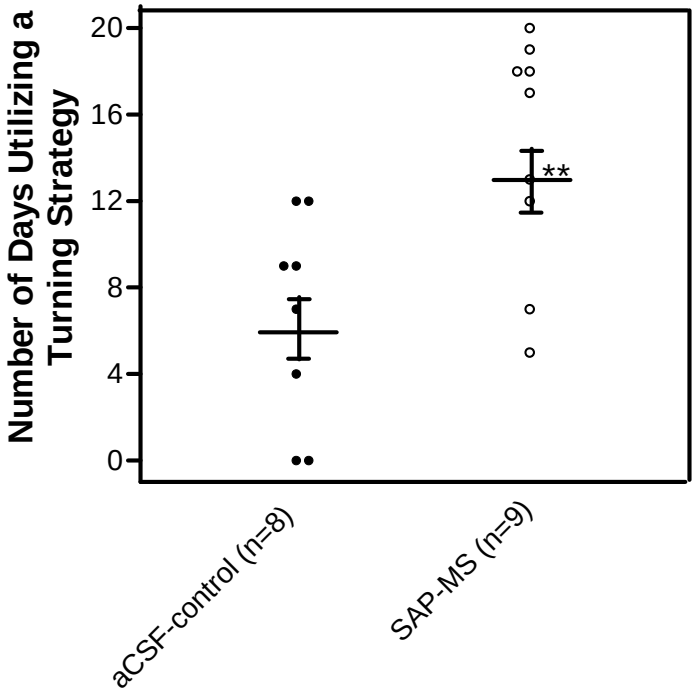


Figure 6. Number of days animals were engaged in a persistent turning strategy during DMP training.

The number of days rats displayed a turning strategy during DMP T-maze training. Line in the middle represents the mean ± s.e.m. Note that SAP treated animals adopted a turning strategy for more days when compared to controls. ** $p < 0.01$ relative to the aCSF treated controls.

To determine whether the percentage of animals that adopted the turning strategy had any significant effect on the analysis of the number of days animals engaged in the turning strategy, those animals that never adopted a turning strategy were excluded. After excluding these animals, there was still a significant difference between SAP treated animals and aCSF treated controls (Fig. 7), suggesting that at least some of the difference was due to an increased utilization of the turning strategy following SAP lesioning of the MS.

To determine whether the differences in the number of days rats were engaged in a turning strategy was enough to explain the effects of treatment differences on acquisition of the DMP task, the days using the turning strategy were subtracted from days required to reach criterion (Fig. 8). Once the days using a turning strategy were subtracted from days to criterion, no significant effect on days to criterion was seen with SAP lesion of the MS ($p = 0.49$). This finding indicated that a significant portion of the effect SAP lesion had on days to criterion was due to the number of days SAP-MS animals were engaged in a turning strategy.

Next, correlations between duration of the turning strategy and ChAT activity in different brain regions were examined (Fig. 9A – 9B). Spearmen correlation of ChAT activity within the hippocampus revealed a significant negative correlation ($p < 0.05$), indicating that as ChAT activity within the hippocampus increased, the number of days the animals engaged in a turning strategy decreased. No relationship was revealed when comparing ChAT activity in the frontal cortex and the number of days animals utilized a turning strategy. The relationship between ChAT activity in the hippocampus and turning strategy was further analyzed by Spearmen correlation within each treatment

group. Within each treatment group (aCSF-controls, SAP-MS) there was no significant relationship between ChAT activity of the hippocampus and turning strategy.

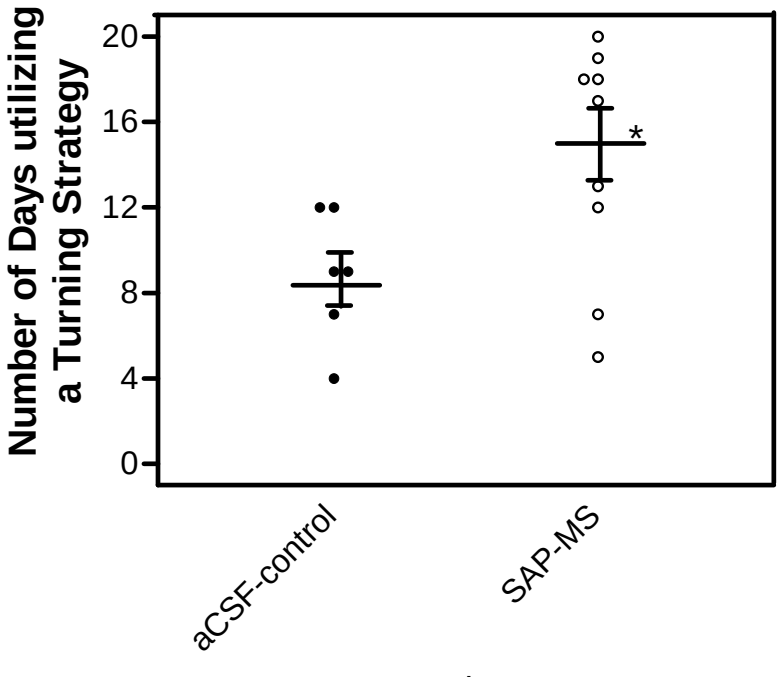


Figure 7. Number of days animals were engaged in a persistent turning strategy excluding those animals which never adopted the learning strategy.

The number of days rats displayed a turning strategy during DMP T-maze training, **excluding those animals that never adopted the strategy**. The line in the middle represents the mean ± s.e.m. Note that SAP treated animals still adopted a turning strategy for more days (14.33 ± 1.81) when compared to controls (8.83 ± 1.24). * $p < 0.05$ relative to the aCSF treated controls.

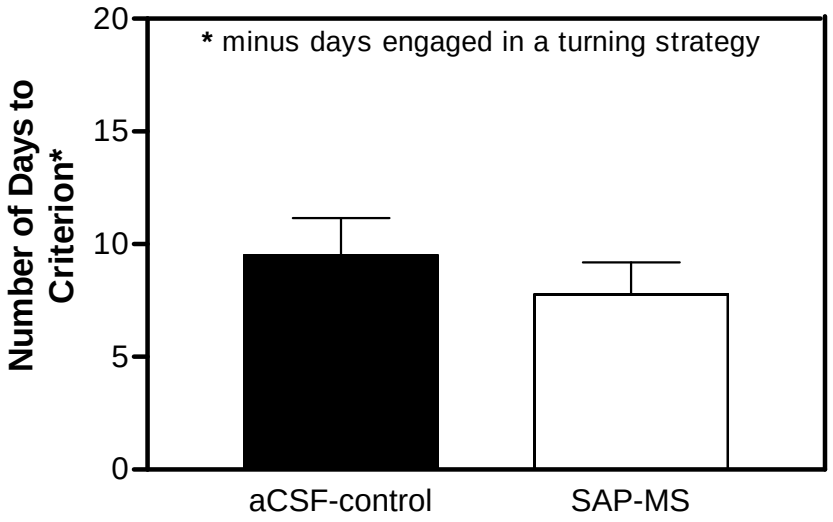


Figure 8. Effect of SAP-lesion of the MS on the number of days to reach criterion in the DMP-task excluding days animals were engaged in a persistent turning strategy. Bar graph represents the days to reach criterion in the DMP task. Top of bar is the mean ± sem. Note that SAP-MS treated animals no longer required significantly more days to reach criterion when compared to controls [p=0.48].

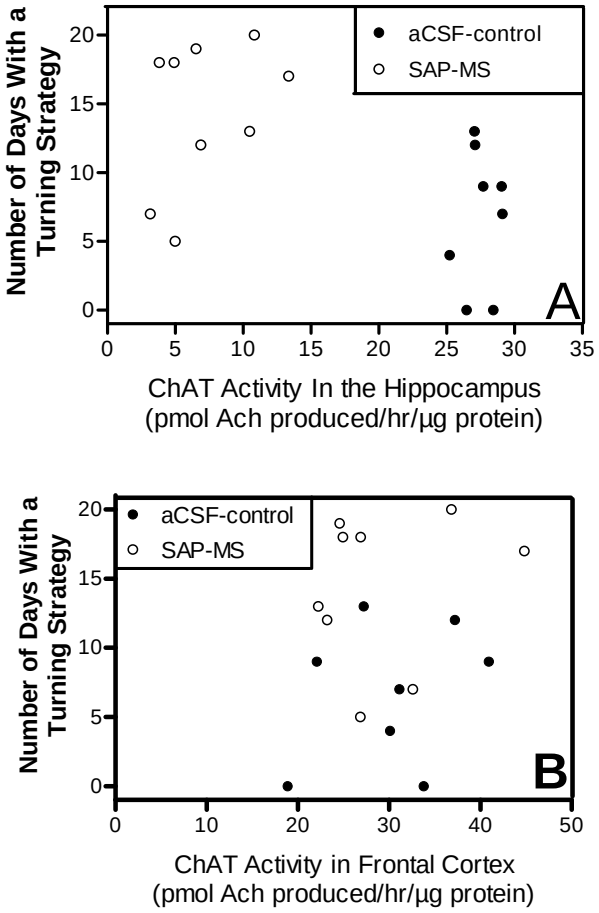


Figure 9A – 9B. Correlation of ChAT activity with the number of days animals adopted a persistent turning strategy.

The correlation of ChAT activity in the hippocampus (panel A) and frontal cortex (panel B) with the number of days animals adopt a turning strategy.

4. Discussion:

Examining learning curves and response patterns (Fig. 2) suggests that the acquisition deficit in the DMP task following SAP lesion of the MS most likely was related to the strategies adopted during training. At the start of training, all groups performed below chance, reflecting the rats' natural tendency for spontaneous alternation (not revisiting a prior location), but after 6 to 9 days of training the rodents reached chance performance. This period of training coincided with the period of training when most animals adopted a persistent turning strategy, always entering either the left or right goal arm during the open choice trial. These findings are similar to observations made by Pych *et al.* who noted that some rats tested on a spontaneous alternation task adopted a persistent turning strategy (197). In the DMP task, use of a persistent turning strategy resulted in enhanced performance early, but with further training the rodents' performance maintained 50% correct. To further improve performance and ultimately reach criterion, the behavioral patterns needed to change, adopting an alternative strategy. Therefore, the number of days to reach criterion was a function of when the animal adopted a persistent turning strategy, the number of days that the animal uses this strategy, and the ability of the animal to deviate from this strategy and adopt a different strategy.

Analysis of the data showed that SAP lesion of the MS had no effect on the likelihood that an animal would adopt a turning strategy (defined as 7/8 turns to the same goal arm) during training of the DMP paradigm. However, SAP lesion increased the number of days that rats utilized the persistent turning strategy before switching to the more successful DMP strategy to reach criterion.

When the number of days rats used a turning strategy was subtracted from number of days rats took to reach criterion, no significant effect of SAP lesion was detected. This result suggests that the effect of SAP lesion on DMP acquisition can be fully explained by the change in response pattern; extended use of a persistent turning strategy; and a delay in changing to the DMP strategy during training. This result also suggests that SAP lesion of the MS has a greater impact on cognitive flexibility, as opposed to spatial working memory.

It was clear that both treatment groups adopted a persistent turning strategy early during training, but what was not clear was whether both groups utilized the same cues to drive this strategy. Once animals adopted the turning strategy the introduction of the probe trial indicated that rotating the maze between forced and open choices failed to disrupt arm choice. This result demonstrated that when rotating the maze, animals still persisted in turning consistently to left or right goal arm. This indicates that neither treatment groups utilized extramaze cues to navigate to the goal arm; but adopted an egocentric (response) strategy that was manifested as persistent turning early in acquisition training.

It was also clear that nearly all animals adopted the more successful DMP strategy, because with extensive training most animals reached criterion. This raised the question of whether both treatment groups eventually would adopt the same strategy to reach criterion (15/16 correct choices). Examining the results of the probe trial after the rats reached criterion indicated that rotating the maze disrupted performance to chance levels. Furthermore, examining the response patterns after rotating the maze indicated that certain animals from each treatment group readopt a persistent turning strategy,

while other animals appeared to select goal arms at random. One of three possibilities was concluded by the time rats reached criterion: 1.) A significant number of animals in each treatment group used extramaze cues to select the correct goal arm. Use of extramaze cues is consistent with an allocentric strategy. Rotating the maze made these animals become confused and they choose arms at random. 2.) Each animal in each group was equally likely to use a persistent turning strategy or extramaze cues to make a selection during the choice phase of learning. 3.) Half of the animals in each group were strongly inclined to use extramaze cues to make their choice and half were strongly inclined to use a persistent turning strategy.

Correlation analysis of ChAT activity vs. days to criterion revealed a negative correlation between the number of days with a turning strategy and the degree of cholinergic activity in the hippocampi for both treatment groups. Note that a negative correlation indicates that a decrease in ChAT activity was associated with an increase in the number of days animals used a turning strategy. This same correlation could not be made within each treatment group. This finding may suggest that when cholinergic afferents to the hippocampus are destroyed, there is a causal relationship between the degree of cholinergic impairment and the delay in shift to an allocentric strategy. This could also suggest that when the cholinergic inputs to one structure are compromised, remaining intact structures may become more influential in determining the navigational strategy used.

It is well accepted that different neuronal circuits underlie different types of learning strategies, and that the activity of specific cholinergic projections can reflect the use of different strategies (for review see 65). It is also well accepted that rodents can use

a variety of learning strategies to acquire T-maze tasks, and that the strategy can change throughout the course of training (203). Rats trained to approach a bated arm in a cross maze initially used a place strategy, but later shifted to a response strategy, indicating that with extensive training a shift in learning strategies can control the animals' behavior (200). This study also showed that inactivation of the hippocampus with lidocaine selectively interfered with a rodents' ability to adopt a place strategy, while inactivation of the caudate nucleus selectively interfered with the adoption of a response strategy. These results suggest that the hippocampus and caudate nucleus are differentially promoting two distinct navigational strategies in the T-maze.

In addition, studies from Gold and colleagues have examined the release of acetylcholine in the hippocampus and other structures as a marker for the involvement of different neuronal systems in specific behavioral responses or cognitive processes. For example, the ratio of acetylcholine released from the hippocampus and caudate nucleus can accurately reflect the use of place and response strategies used during training (204). When rats were trained on a plus maze that could be learned using either a response or place strategy, the rats that used the response strategy displayed a lower ratio of acetylcholine release in the hippocampus/striatum than rats that used the place strategy (204). Furthermore, Gold *et al.* (67) showed that in the cross maze the cholinergic hippocampal system was engaged first before the striatum was activated, and that the hippocampus was important for place learning. Later in training, although the hippocampus remained activated, the striatum was also activated in a manner that enabled the use of a response strategy to solve the maze. These results suggest that the behavioral flexibility needed to change learning strategies was associated with

cholinergic activity of the dorsal striatum as opposed to cholinergic activity of cortical structures. Further studies are needed to determine whether cholinergic activity in other neuronal systems can affect the utilization of different nagivational strategies in the DMP paradigm.

In another study, rats tested on a food-reward spontaneous alternation task initially used a spatial working memory strategy, but later shifted to a persistent turning strategy (197). Again, as rats adopted the persistent turning strategy, the ratio of hippocampal/striatal ACh released steadily decreased. Recently, Pych *et al.* showed that ACh release was greater in the striatum during training on a response food-reward task as compared to the same place food-reward task (67). The levels of ACh increased in the place and response tasks when extramaze cues were available. When these cues were minimized the levels of ACh were significantly lower during training with a response task. This finding demonstrated a strong relationship between the ratios of cholinergic activity in different neuronal systems and the adoption of different learning strategies. These results also provide a possible explanation for the extended reliance on the persistent turning- response strategy following septo-hippocampal cholinergic lesions.

In summary, the data show that SAP lesion of the MS impaired acquisition of the DMP task, and that this effect was primarily due to a delayed shift in navigational strategy during training. Specifically, septo-hippocampal cholinergic lesions appeared to impair the ability of animals to adopt an allocentric place strategy needed to reach criterion.

D. Effects of DU-14 treatment on spatial acquisition of medial septum cholinergic lesioned rats.

1. Introduction

There is now good evidence that the metabolism of DHEA and its sulfated form occurs bi-directionally within the CNS, with DHEAS being desulfated via the enzyme steroid sulfatase (108). DU-14 is a potent non-estrogenic irreversible inhibitor of steroid sulfatase. A single dose of DU-14 (30 mg/kg) was able to inhibit 95.2% of the steroid sulfatase activity within the liver (116). Furthermore, this treatment was able to significantly lower steroid sulfatase activity within the brain but at a substantially lower rate (14.8% reduction in activity) (116). Chronic treatments with DU-14 (30 mg/kg for 15 days i.p.) in rodents increased plasma concentrations of DHEAS, while decreasing plasma DHEA (113). Furthermore, this same treatment increased whole brain levels of DHEAS.

The administration of DU-14 (30 mg/kg, i.p.) has been shown to increase hippocampal ACh release, a result which supports the findings that increased DHEAS levels in the brain augment the levels of ACh within the hippocampus (157). Additionally, chronic pretreatment with DU-14 (30 mg/kg for 15 days i.p.) attenuated scopolamine induced spatial memory impairment in the passive avoidance paradigm and Morris water maze (116, 158). In the Morris water maze not only did DU-14 reverse the scopolamine induced amnesia, but enhanced the performance of control animals (116). In agreement with the GABA antagonistic action of DHEAS, these findings suggested that DHEAS rather than DHEA was responsible for memory enhancement. Since all of these experiments administered the steroid sulfatase inhibitor through an i.p. route, it

remains unclear whether the observed effects were caused by actions of the inhibitor at the peripheral or central level.

We hypothesized that DU-14 will attenuate the cognitive deficits associated with lesion of septal-hippocampal cholinergic projections. DU-14 is one possible treatment which could reverse impairments in the DMP task associated with SAP lesioning of the MS. Studies have shown that DU-14 does, in fact, increase DHEAS in both plasma and whole brain, elevates hippocampal ACh, and enhances passive avoidance retention as well as performance in the Morris water maze (113, 197, 205). Whether the memory-enhancing effects associated with DU-14 require an intact septal-hippocampal tract or if DU-14 can improve spatial acquisition in SAP lesioned animals remains unknown. We predict that chronic treatments of DU-14 will improve performance of both SAP treated and aCSF treated control animals in the DMP T-maze task. Where the cognitive enhancing effects associated with DU-14 are due specifically to changes in Ach release in the hippocampus and cortex is currently unknown. One possibility is that DU-14 may also affect non-cholinergic systems in ways which can compensate for the loss of muscarinic cholinergic activity. If so, then steroid sulfatase inhibitors could potentially provide effective agents for the treatment of cognitive dysfunctions associated with a loss of basal forebrain cholinergic neurons such as in AD. This study will help determine the mechanisms by which steroid sulfatase inhibitors enhance learning and memory processes, and may ultimately lead to the development of a new class of therapeutic agents for the treatment of cognitive decline associated with aging and AD.

2. Methods

Immunolesioning of the cholinergic neurons of the MS with SAP was performed as documented in Experiment A. Acclimation and acquisition of the DMP T-maze task was performed as documented in Experiment B. Following behavioral testing ChAT activity in the hippocampus and frontal cortex was determined for each animal as described in Experiment A. SAP treated animals that did not show selective decrease in ChAT activity within the hippocampus were eliminated from the study.

i. (P-o-sulfamoyl)-N-tetradeconoyl tyramine (DU-14) Treatment:

Fourteen days after SAP or aCSF infusion, the animals were randomly separated into DU-14 treatment group and controls. DU-14 was suspended in corn oil. DU-14 (30 mg/kg, i.p.) or vehicle (oil) was administered, via an IP injection, 4 hours following the testing procedure. This treatment regiment was continued until the animal met a criterion of 15 correct choices out of 16 consecutive trial pairs. So that introduction of an aversive stimulus would not interfere with testing, administration of DU-14 4 hours following testing was chosen. The concentration and route of administration of DU-14 (30 mg/kg, i.p.) used in this study has been shown to increase DHEAS in both plasma and whole brain, elevates hippocampal ACh, and enhances passive avoidance retention as well as performance in the Morris water maze (113, 197, 205).

ii. Statistical Analysis:

Days to criterion were analyzed using a parametric one-way ANOVA with Newman-Keuls post hoc test. To compare treatment effects during different stages of

acquisition, performance data was blocked into 3-day periods of training. After an animal had reached criterion a value of 93.75% (15/16) was recorded for performance on subsequent days. Performance during acquisition testing was analyzed using two-way ANOVA for overall effects of block and treatment and one-way ANOVA with Newman-Keuls post hoc test for treatment effects within blocks.

During the course of DMP training it was observed that throughout early stages of training, many animals adopt a turning strategy whereby they consistently turned to the right or left goal arm of the maze. To quantify this observation, we counted the total number of days the animals utilized the strategy during the testing process. Any animal that entered 7 out of 8 times into the same goal arm was defined as using a turning strategy. A one-way ANOVA with Newman-Keuls post hoc test was used to analyze the number of animals that adopted this strategy and also the number of days that an animal utilized the strategy. Both the rate of performance and days to reach criterion in the DMP t-maze task was determined to be parametric. All analyses were performed using GraphPad Prism 3.0.

3. Results

Of the original 56 animals, all but three animals failed to reach criterion on the delayed matching to position (DMP) task or displayed a preference for one goal arm of the T-maze (side bias) and were excluded from the study. Two of the three animals were aCSF-vehicle treated control animals while the other was a SAP-MS DU-14 treated animal. Analysis of the remaining animals (12 aCSF-vehicle, 14 aCSF-DU-14, 14 SAP-MS vehicle, and 13 SAP-MS DU-14 treated animals) indicated that SAP treated animals

required more days to reach criterion and treatments with DU-14 further increased the number of days required for the animals to reach criterion (Fig. 10). Analysis of days to criterion by a Newman Keuls one-way ANOVA found a significant effect of DU-14 treatment in SAP-MS animals ($p < 0.05$), indicating that SAP-MS vehicle treated animals differed significantly from the SAP-MS DU-14 treated animals with SAP-MS lesioned animals taking a significantly longer time to complete the maze compared to treatment controls. Furthermore, there was a significant effect of SAP lesioning in both treatment groups ($p<0.05$). aCSF vehicle treated animals took an average of 14.33 ± 3.2 days while the SAP-MS vehicle treated animals took an average of 17.86 ± 3.527 to complete the DMP task. Treatment with DU-14 significantly increased the number of days to criterion for SAP-MS animals while having no significant impact on the days to criterion for aCSF treated controls. SAP-MS vehicle treated animals took an average of 17.86 ± 3.527 to complete the DMP task while treatment with DU-14 increased days to criterion to an average of 21.15 ± 4.451.

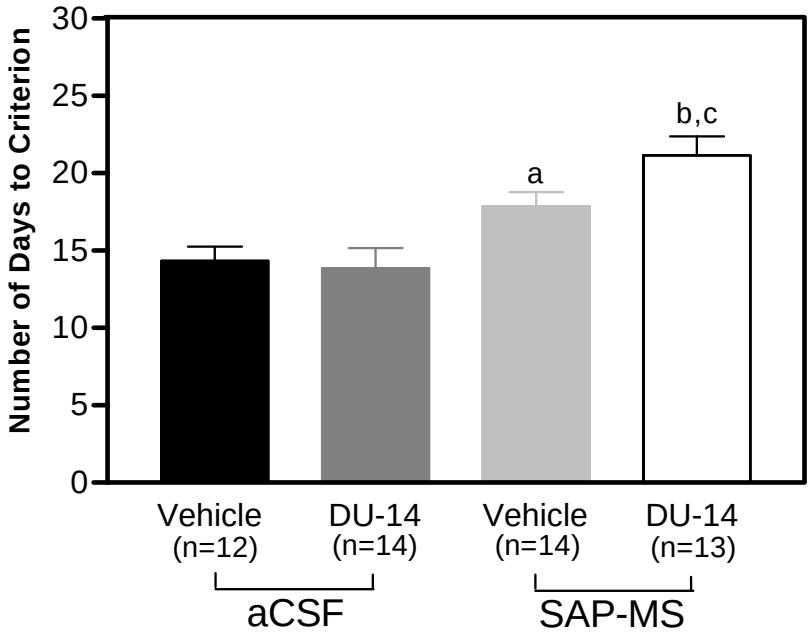


Figure 10. Effects of DU-14 treatment on the number of days to reach criterion in the DMP T-maze task.

Bar graph representing the effect of DU-14 treatment on spatial acquisition of MS cholinergic lesioned rats. Top of bar represents the mean number of days to reach criterion ± sem. DU-14 treated SAP-MS animals required significantly more days to reach criterion than the SAP vehicle treated animals. DU-14 had no effect on days to criterion of aCSF control animals. a: $p < 0.05$ relative to the aCSF-vehicle treated animals. b: $p < 0.01$ relative to the aCSF-DU14 treated animals. c: $p < 0.05$ relative to SAP-MS vehicle treated animals.

Examination of the learning curves (Fig. 11) show that all animals performed at similar, below chance, levels at the start of DMP training. SAP-MS vehicle treated animals improved at a slower rate during training when compared to aCSF vehicle treated controls. SAP-MS DU-14 treated animals improved at the slowest rate. By day 21 of training, all aCSF vehicle treated animals and all but one aCSF DU-14 treated had reached criterion. On the other hand, only six of 13 SAP-MS DU-14 treated animals had reached criterion. By day thirty all animals reached criterion. Analysis of the learning curves by two-way ANOVA demonstrated a significant effect of "Treatment" ($F[27,88] = 6.88$, $p < 0.0001$), a significant effect of "Block" ($F[9,5425] = 425.90$, $p < 0.0001$), and a significant 'Treatment' X 'Block' interaction ($F[27,88] = 6.88$, $p < 0.0001$). A separate analysis of performance within block revealed aCSF vehicle treated animals performed significantly better than SAP vehicle treated animals during blocks 4 and 5 of training. aCSF DU-14 treated animals performed significantly better during blocks 3 and 4 when compared to aCSF vehicle treated animals. SAP-MS vehicle animals performed significantly better during blocks 4 and 6 when compared to SAP-MS DU-14 treated animals.

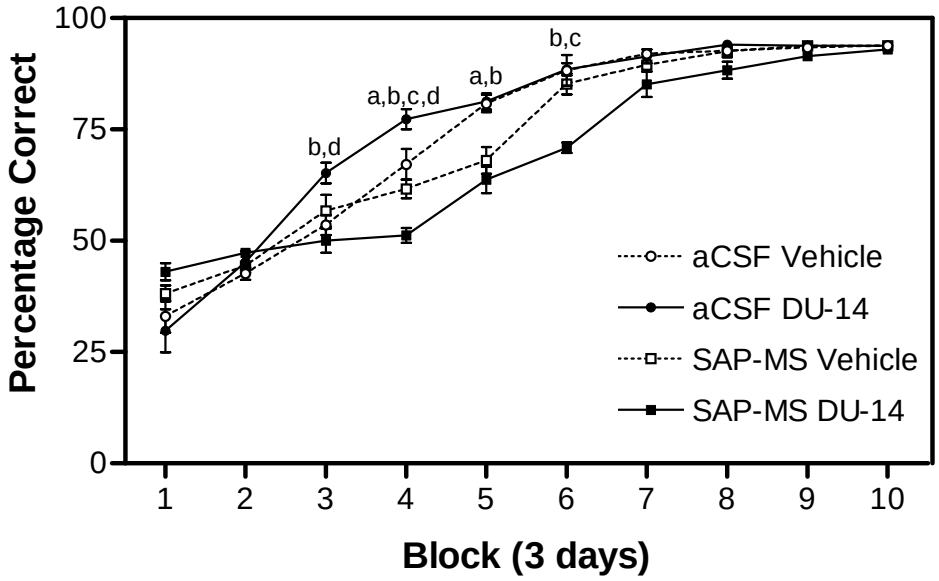


Figure 11. Effects of DU-14 treatment on the rate of acquisition in the DMP T-maze task.

The effects of DU-14 treatment on the rate of DMP acquisition for SAP-MS lesioned rats. Points represent the mean percentage correct for each treatment group during a 3 day period of training. Note that all groups showed improved performance over time; however during blocks 4 and 6 the performance of the SAP-MS DU-14 treated animals was significantly worse than the corresponding SAP-MS vehicle. Also during blocks 3 and 4 aCSF vehicle treated animals performed significantly worse than aCSF DU-14 treated animals. a: $p < 0.005$ for aCSF vehicle treated animals relative to SAP-MS vehicle treated animals. b: $p < 0.005$ for aCSF DU-14 treated animals relative to SAP-MS DU-14 treated animals. c: $p < 0.005$ for SAP-MS vehicle treated animals relative to SAP-MS DU-14 treated animals. d: $p < 0.005$ for aCSF vehicle treated animals relative to aCSF DU-14 treated animals.

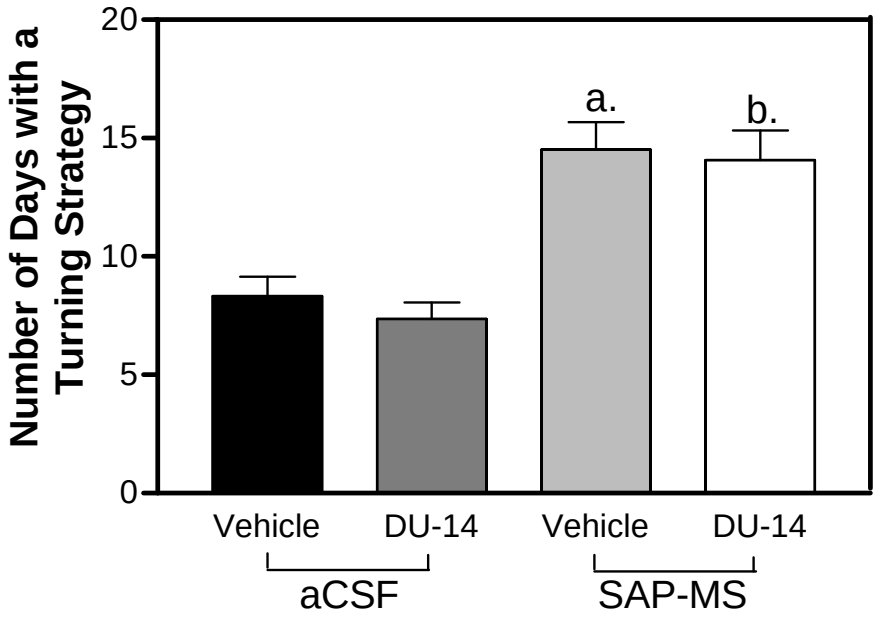


Figure 12: Effects that the daily injections of DU-14 had on the utilization of a persistent turning strategy.

The number of days rats displayed a turning strategy during DMP T-maze training. The top of the bar represents the mean number of days using a turning strategy ± s.e.m. Note that SAP-MS vehicle treated animals adopted a turning strategy for more days compared to aCSF vehicle treated controls. Also note that there was no significance difference between aCSF DU-14 and aCSF vehicle. Furthermore SAP-MS DU-14 animals did not utilize a turning strategy for significantly more days when compared to vehicle treated SAP-MS animals. a. $p < 0.001$ relative to the aCSF vehicle and DU-14 treated controls. b. $p<0.001$ relative to aCSF vehicle and DU-14 treated animals.

As mentioned above, we observed that during early stages of training many of the animals adopted a turning strategy. There was no significant difference in the number of animals in each treatment group which adopted the persistent turning strategy. Furthermore, there was no significant difference in the number of days before each of the treatment groups adopted the turning strategy. The number of days that animals engaged in the turning strategy was significantly ($p<0.001$) greater for rats with SAP lesions (Fig. 12). SAP-MS vehicle treated animals engaged in a turning strategy for 14.50 ± 1.18 days while aCSF vehicle treated controls engaged in this strategy for 8.33 ± .80 days. SAP-MS DU-14 treated animals engaged in a turning strategy for 14.08 ± 1.25 days while aCSF DU-14 treated animals engaged in this strategy for 7.35 ± 0.71 days. However, there was no significant difference in the number of days SAP-MS or aCSF animals used the turning strategy following DU-14 treated. To determine whether the percentage of animals that adopted the turning strategy had any significant effect on the analysis of the number of days animals engaged in the turning strategy, those animals that never adopted a turning strategy were excluded. After excluding these animals, there was still no significant difference in the number of days SAP treated animals and aCSF treated controls used the turning strategy following DU-14 treatment. This suggests that the difference in days to criterion between SAP-MS vehicle and SAP-MS DU-14 can not be explained by an increased utilization of the turning strategy following DU-14 treatment.

4. Discussion

Previous investigations found that in addition to decreasing scopolamine induced impairment in the Morris water maze, DU-14 (30 mg/kg i.p.) was able to enhanced

performance in control animals (152). The mechanism of action for the enhanced cognitive function associated with steroid sulfatase inhibitors may be due to an increase in sulfated neurosteroids such as DHEAS and pregnenolone sulfate (PS) (113, 205). DHEAS and PS are allosteric antagonists of the GABAa receptor. Since GABAergic neurons maintain an inhibitory tone on cholinergic neurons, DHEAS may disinhibit cholinergic firing resulting in enhanced activation of central muscarinic and nicotinic receptors. Enhance activation of these receptors especially in the hippocampus could facilitate spatial learning and memory. Another possible mechanism for the effect of DU-14 on memory could be through increased hippocampal Ach release (205).

Examination of the ability of DU-14 to attenuate impairment in the rate acquisition of the DMP task following SAP-MS lesions demonstrated that DU-14 treatment further impaired acquisition for the SAP-MS treated rodents. Specifically, lesioned rats that received DU-14 had an increase in the number of days to reach criterion. This was a result of decreased performance during blocks 4 and 6 when compared to control treated SAP-MS lesioned animals. These findings opposed the initial hypothesis, that chronic pretreatment with DU-14 would improve acquisition of the DMP paradigm for animals with SAP lesion of the MS. One possible explanation for this result may be that GABAergic neurons play an important role in cognition aside from modulating cholinergic tone (206). Decreased acquisition of the DMP task following SAP lesion of the MS may be further impaired by the loss of GABAergic neurotransmission via the physiologic antagonistic properties of DU-14. DU-14 may be able to reverse scopolamine induced amnesia by increasing the cholinergic tone within the hippocampus. Increased Ach release would then compete with scopolamine for

binding sites on muscarinic receptors. However, unlike scopolamine induced amnesia, SAP lesion of the MS permanently destroys cholinergic afferents of the hippocampus. Therefore, with SAP lesioned animals, treatments with DU-14 may be unable to increase cholinergic activity within the hippocampus, resulting in no beneficial effect on memory.

DU-14 treatment could increase Ach released in other neuronal systems involved in different learning strategies. As stated above, Gold *et al.* (67) showed that relative levels of Ach in the hippocampus and striatum were associated with different learning strategies. DU-14 treatment in SAP lesioned animals could increase acetylcholine levels in the striatum while hippocampal levels remained low, resulting in an ACh ratio favorable toward an egocentric response strategy. This seems unlikely since there was no significant difference in the number of days DU-14 treated SAP-MS lesioned rats utilized the persistent turning strategy when compared to control treated SAP-MS rats.

Another possible reason for the diminished performance of SAP-MS animals following DU-14 treatment may be changes in the theta rhythms in the hippocampus. Theta rhythms are one of several characteristic electroencephalogram waveforms associated with various states of sleep and wakefulness. Theta rhythms have been implicated in spatial navigation and some forms of learning and memory, especially those associated with the temporal lobes and the hippocampus (207). Theta rhythm activity is also manifested during certain short-term memory tasks (208). Studies suggest that these waves reflect the readiness of the hippocampus to process incoming signals (209). Furthermore, theta oscillations have been correlated to different voluntary behaviors (exploration, spatial navigation, etc.), suggesting that the theta oscillations may reflect the integration of sensory information with motor output (210, 211). Treatments with DU-14

may alter theta oscillations and therefore diminish the ability to integrate the sensory information from the DMP task with the motor output. SAP-MS animals treated with DU-14 may have a diminished ability to use the extra-maze cues to guide their behavior resulting in diminished rate of acquisition of the DMP task and increased days to criterion.

In summary, the results suggest that the cognitive enhancing and anti-amnestic effects of DU-14 relay some what on an intact cholinergic afferent to the hippocampus. Furthermore, this may suggest that the cognitive enhancing and anti-amnestic effects of DU-14 are due to its ability to increase concentration ACh within the hippocampus. Since neither plasma nor whole brain concentrations of DHEAS were examined we cannot correlate findings to DHEAS levels. Prior studies have found that acute administration of DHEAS increase the release of ACh in the rat hippocampus, with corresponding enhancement of passive avoidance retention. It has yet to be determined if acute administration of DHEAS alone will improve spatial acquisition of SAP-MS lesioned rats.

E. Effects of DHEAS treatment on spatial acquisition of medial septum cholinergic lesioned rats.

1. Introduction

DHEA and its sulfated ester DHEAS have gained significant interest in the field of neuroscience due to two findings: a strong age associated decline of the steroid in humans and the demonstration of DHEA(S) metabolism and action in the rodent brain. DHEA(S) concentrations in the rodent brain far exceed peripheral concentrations and are independent of adrenal synthesis (108, 109).

Flood *et al.* were the first to show the memory-enhancing effects of DHEA and DHEAS, in both young (120-122) and old (123) mice using a foot shock paradigm. In their extensive studies, multiple routes of administration (i.c.v., s.c., oral) were utilized, all leading to an inverted U-shaped dose response curve for memory-enhancing effects. Melchior and Ritzmann (124) demonstrated that when administered i.p., both DHEA and DHEAS enhanced short-term working memory as assessed with a T-maze paradigm. Again, an inverted U-shaped dose response curve was observed. Another group of experiments demonstrated that DHEAS enhanced memory when given before or directly after training, but not when administered before retention; suggesting that DHEAS enhanced the storage and/or consolidation of the learned paradigm but not retrieval (125).

Other studies have investigated the anti-amnestic properties of DHEA(S) using a variety of amnestic agents. Flood *et al.* were again the first to show the anti-amnestic properties of excitatory neurosteroids using multiple routes of administration (120-122). Maurice *et al.* confirmed the antiamnestic effect of DHEA and DHEAS in mice utilizing multiple memory paradigms (Y maze, water maze, passive avoidance) with multiple amnestic agents (126-128). Moreover, Maurice *et al.* demonstrated that DHEAS had effects on learning and/or storing information but no effect on recall of the same information (127).

DHEA(S) has memory enhancing and anti-amnestic effects on several brain locations associated with memory function, suggesting a global effect rather than actions limited to particular structures. Paradigms utilized to show the memory enhancing effects of DHEA(S) rely on different neuronal structures, especially the hippocampus, the amygdala and frontal cortical regions (for review see 129).

Since DHEA(S) concentrations decrease significantly with age in humans and multiple beneficial effects of DHEAS have been documented in rodents, many studies have investigated the relationship between DHEA(S) and cognition in humans. High-functioning elderly patients, as defined by cognitive and functional examinations, possess higher DHEAS levels compared to patients in the median or lower functioning groups (132, 133). Recent studies have shown that among Alzheimer's disease patients, those with higher concentrations of plasma DHEAS performed better on tests of association, digit span, and mini mental status exams when compared to those with lower levels of this neurosteroid (137).

There are several possible pathways by which neurosteroids enhance memory function. The GABAergic neurons of the nucleus accumbens are known to synapse upon cholinergic neurons of the MS which then form the major cholinergic projections to the hippocampus (155). Therefore, DHEA(S) may enhance memory by disinhibiting the cholinergic neurons of the MS and increasing concentrations of acetylcholine in the hippocampus. In support of this hypothetical pathway, peripheral administration of DHEAS enhanced hippocampal ACh release in vivo, and this enhancement occurred in a dose-dependent manner (155). In further support of this hypothesis, Yoo *et al.* demonstrated a dose dependent increase in hippocampal long term potentiation following treatment with DHEAS (156). Where the cognitive enhancing effects associated with DHEAS are due specifically to changes in Ach release in the hippocampus and cortex is currently unknown. One possibility is that DHEAS may also affect non-cholinergic systems in ways which can compensate for the loss of muscarinic cholinergic activity. If so, then DHEAS could potentially provide effective agents for the treatment of cognitive

dysfunctions associated with a loss of basal forebrain cholinergic neurons such as in AD. In this study the approach is to produce selective lesions of the cholinergic neurons projecting to the hippocampus using SAP. We will then assess the ability of DHEAS to enhance learning and memory in the DMP task. This study will reveal whether DHEAS can attenuate learning and memory in the DMP T-maze task under conditions when most of the cholinergic projections to the hippocampus have been eliminated. DU-14 is a non-selective steroid sulfatase inhibitor preventing the metabolism of many different sulfated neurosteroids (DHEAS, PS, etc.); therefore results from Experiment B cannot be related to the action of a single neurosteroid. This study differs from Experiment D in that only DHEAS will be administered and examined for its ability to attenuate performance of SAP-MS treated animals in the DMP task.

2. Methods

Immunolesioning of the cholinergic neurons of the MS with SAP was performed as documented in Experiment A. Acclimation and acquisition of the DMP T-maze task was performed as documented in Experiment B. Analysis of the DMP behavior data was performed as documented in Experiment D. Following behavioral testing ChAT activity in the hippocampus and frontal cortex was determined for each animal as described in Experiment A. SAP treated animals that did not show selective decrease in ChAT activity within the hippocampus were eliminated from the study.

i. Dehydroepiandrosterone sulfate (DHEAS) Treatment:

Fourteen days after SAP or aCSF infusion, the animals were randomly separated into various DHEAS treatment groups and controls. Various concentrations of DHEAS (0, 3, 10, 20 mg/ml) were dissolved in sterile saline. DHEAS doses were made fresh daily and protected from light. DHEAS (3, 10, 20 mg/kg), or vehicle (saline) was administered, via an IP injection, 30 mins prior to daily training until the animal reached a criterion of 15 correct choices out of 16 consecutive trial pairs. Due to the potential of DHEAS to be converted to DHEA and the short half-life of DHEA 30 min prior to testing was chosen. A daily IP injection was utilized to better replicate a potential treatment regiment which could be utilized in humans. Prior studies demonstrated that the same concentrations of DHEAS used in the current study were able to significantly increase hippocampal Ach release 60 minutes following an i.p. injection (113). DHEAS also reversed scopolamine induced amnesia in a dose-dependent manner (113, 152, 205). It should be noted that the optimal dose of DHEAS for reversing scopolamine amnesia (20 mg/kg) was much lower than the dose which produced the maximal release of acetylcholine from the hippocampus.

3. Results

Of the original 67 animals, five animals failed to reach criterion on the DMP task or displayed a side bias and were excluded from the study. One animal from the following groups failed to complete the DMP task: aCSF vehicle, aCSF DHEAS (3 mg/kg), aCSF DHEAS (10 mg/kg) animals while the other two animals were SAP-MS DHEAS (10 mg/kg) treated. Analysis of the remaining animals indicated that DHEAS

treatment failed to improve the days to criterion for either the aCSF or SAP-MS treated animals (Fig. 13 & 14). Table 2 summarizes the effects of DHEAS treatment on days to criterion.

Table 2: Summary of days-to-criterion data following daily DHEAS injections.

Lesioning	DHEAS Treatment (mg/kg)	Days to Criterion	S.E.M	Number
aCSF	0	14.00	1.07	9
aCSF	3	14.88	1.30	8
aCSF	10	17.00	1.54	8
aCSF	20	12.67	1.77	9
SAP-MS	0	17.38	3.34	8
SAP-MS	3	15.83	4.07	6
SAP-MS	10	22.00	4.24	7
SAP-MS	20	16.22	5.95	9

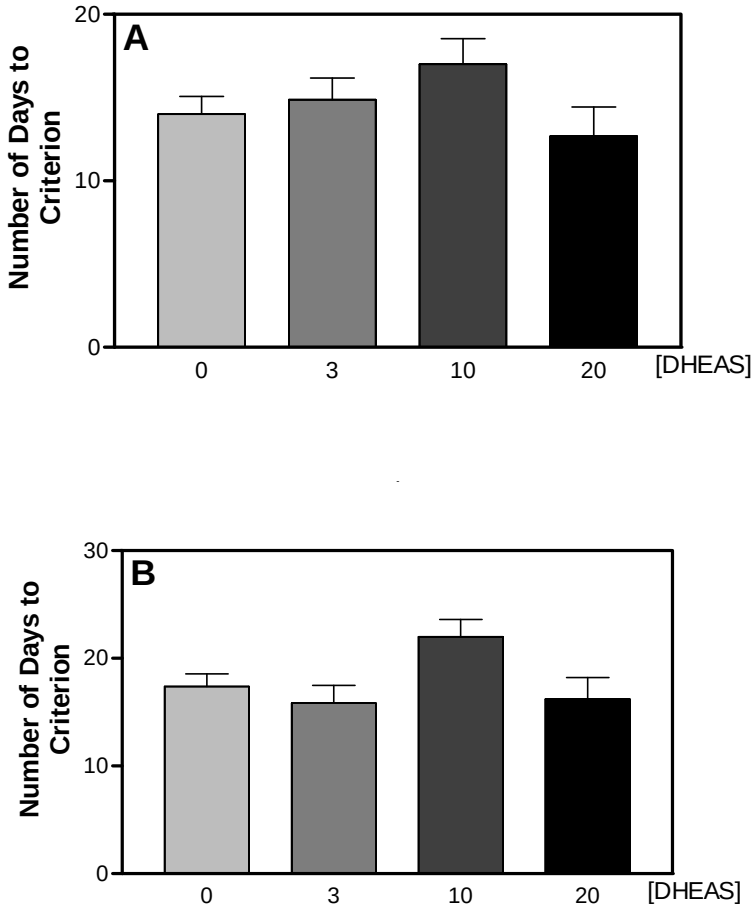


Figure 13. Effects of differing DHEAS treatments on the number of days to reach criterion in the DMP T-maze task.

Bar graph representing the effects of different DHEAS treatments on days to criterion in the DMP task for both aCSF control animals (panel A) and SAP-MS animals (panel B). Top of bar represents the mean number of days to reach criterion ± sem. There was no significant effect of DHEAS treatment on days to criterion.

Examination of the learning curves (Fig. 14A &14B) show that all animals performed at similar, below chance, levels at the start of DMP training. By day thirty all animals had reached criterion. There were minimal effects on the rate of performance with DHEAS treatments for either aCSF or SAP-MS treated animals. Analysis of the learning curves for aCSF rats treated with DHEAS (fig. 14A) by two-way ANOVA demonstrated a significant effect of "Block" ($p < 0.0001$), but no significant effect of "Treatment" ($p = 0.077$) or "Treatment" X "Block" interaction ($p = 0.43$). A post-hoc test showed no significant difference between aCSF DHEAS treatment groups for any of the blocks.

Analysis of the learning curves for SAP-MS rats treated with DHEAS (Fig. 14B) by two-way ANOVA demonstrated a significant effect of "Block" ($p < 0.0001$) and "Treatment" ($p < 0.0001$), but no "Treatment" X "Block" interaction ($p = 0.41$). A separate analysis of performance within a block revealed SAP vehicle treated animals performed significantly better than SAP DHEAS (10mg/kg) treated animals during blocks 1 through 3 of training.

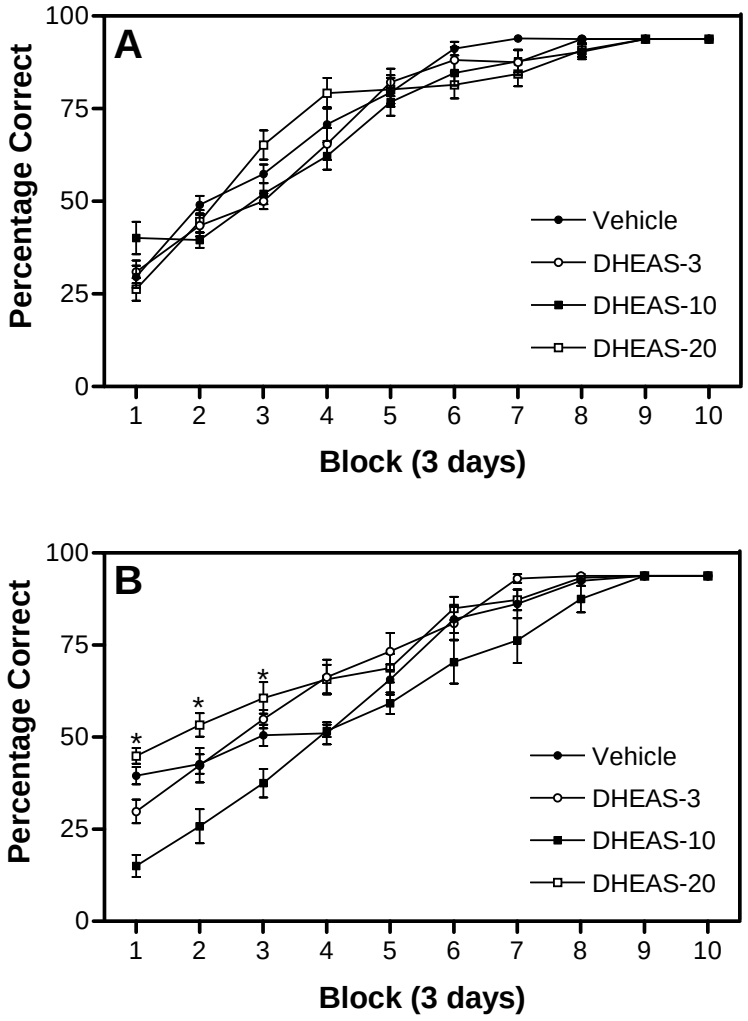


Figure 14. Effects of differing DHEAS treatments on the rate of acquisition in the DMP T-maze task.

Learning curve showing effects of DHEAS treatment on the rate of DMP acquisition for aCSF (panel A) and SAP-MS (panel B) lesioned rats. Points represent the mean percentage correct for each treatment group during a 3 day period of training. DHEAS had no significant effect on the performance of aCSF animals (panel A). SAP animals treated with DHEAS (10mg/kg) significantly impaired performance during blocks 1-3 (panel B). * $p < 0.01$; when comparing DHEAS (10mg/kg) to vehicle treated animals.

4. Discussion

Examination of learning curves and days to criterion data found that differing concentrations of DHEAS treatment had no effect on acquisition of the DMP task for either control or SAP lesioned animals. Specifically, DHEAS treatments did not affect the number of days rats required to reach criterion and had no effect on performance within any of the training blocks. These findings did not support the initial hypothesis that DHEAS would attenuate the cognitive deficits associated with SAP lesions of the MS.

Prior studies demonstrated that the same concentrations of DHEAS used in the current study were able to significantly increase hippocampal acetylcholine release 60 minutes following an i.p. injection (113). DHEAS also reversed scopolamine induced amnesia in a dose-dependent manner (113, 152, 205). It should be noted that the optimal dose of DHEAS for reversing scopolamine amnesia (20 mg/kg) was much lower than the dose which produced the maximal release of acetylcholine from the hippocampus. However, unlike with scopolamine induced amnesia, SAP lesion of the MS permanently destroyed the cholinergic afferents to the hippocampus. The current results suggest that the memory enhancing effects of DHEAS are some what dependent on an intact septo-hippocampal tract. However, the rats with an intact septo-hippocampal tract were also unaffected by DHEAS administration in the DMP task. This result suggests one of two possibilities, a ceiling effect where control animals function at maximum efficiency for acquisition of the DMP task, or that the DMP task is not an appropriate test for demonstrating the memory enhancing effects of DHEAS. This second explanation is unlikely since Melchior and Ritzmann (124) demonstrated that when administered i.p.,

both DHEA and DHEAS enhanced short-term working memory as assessed with a T-maze paradigm. A prior study (Experiment C) suggests that the utilization of different spatial learning strategies in the DMP T-maze task is greatly affected following SAP lesioning of the MS. These results may suggest that DHEAS is unable to change the utilization of these learning strategies adopted by these rats during acquistion. Other studies using the Morris water maze could better assess the effects of DHEAS on spatial working memory.

Flood *et al.* were the first to show the memory-enhancing effects of DHEA and DHEAS, in both young (120-122) and old (123) mice using a foot shock paradigm. In their extensive studies, multiple routes of administration (i.c.v., s.c., oral) were utilized, all leading to an inverted U-shaped dose response curve for memory-enhancing effects. It is possible that the concentrations of DHEAS used in this study are unable to enhance memory. In support of this conclusion, there was no significant change in the DMP performance for aCSF treated controls. However, prior studies demonstrated that the same concentrations of DHEAS used in the current study were able to significantly increase hippocampal Ach release 60 minutes following an i.p. injection (113). Furthermore, it has been demonstrated that 20 mg/kg is the optimal dose of DHEAS for reversing scopolamine amnesia in the Morris water maze (113, 152, 205).

Surprisingly, SAP treated rats failed to show impaired performance in the DMP task compared to sham controls. This result may have been due to enhanced arousal associated with the injection procedure for DHEAS and vehicle. One must consider the potential of enhanced arousal with an i.p. drug administration and should alter the treatment regiment to minimize this complication. Administration of DHEAS through an

implanted osmotic pump or a few hours before or after training may reveal its potential to attenuate learning and memory impairments associated with selective loss of cholinergic neurons projecting to the hippocampus.

F. Effects of aversive stimulus on spatial acquisition of medial septum cholinergic lesioned rats.

1. Introduction

In experiment A and B we showed that SAP can be used to produce selective cholinergic lesions in the MS, resulting in a marked decrease in markers of hippocampal cholinergic function, as well as significant impairment in the acquisition of a DMP T-maze task. Other investigations, though, have reported only limited effects of MS SAP lesions in other spatial tasks. For example, Dornan *et al.* (179) reported that a selective reduction of cholinergic transmission in the basal forebrain was by itself insufficient to account for the functional impairments in spatial learning of rats using a Morris water maze paradigm. Baxter et al. (180, 181), utilizing a Morris water maze task reported similar findings.

These conflicting results may be the consequence of the environment and stress associated with the particular task. For example, Sandi et al., found that lowering water temperature from 25 to 19 degrees Celsius resulted in both increased post training corticosterone levels and enhanced acquisition and retention in the Morris water maze (206). Moreover, emotional and stressful experiences, via the activation of specific hormonal and brain systems, alter learning and memory processes (159, 160, 212). Stress, depending on intensity and duration, can either facilitate or impair cognitive functions, particularly spatial learning and memory performance (162). Short periods of

a mild stressor can enhance acquisition of certain spatial learning tasks (36, 163), while longer periods of stress have impaired performance in a variety of spatial tasks (165, 213). Therefore, we hypothesize that the effects of basal forebrain cholinergic lesions on acquisition of a spatial task may have different outcomes as a consequence of the differing levels of stress inherent to the tasks. A number of studies have shown that intraperitoneal injection of saline acts as a mild stressor, resulting in modest elevations in plasma corticosterone (214, 215). In the present study, we evaluated whether daily introduction of this mild aversive stimulus prior to training would alter the effect of septal cholinergic lesions on acquisition of a DMP task. Specifically, we predicted that introduction of the stressor would reduce the impairment produced by septal cholinergic lesions, consistent with the relative lack of effect of SAP lesions on acquisition of the more stressful Morris water maze task previously described.

2. Methods

Selective immunolesioning of the cholinergic neurons of the MS with SAP was performed as documented in Experiment A. Acclimation and acquisition of the DMP T-maze task was performed as documented in Experiment B. Analysis of behavior data was performed as documented in Experiment D. Following behavioral testing ChAT activity in the hippocampus and frontal cortex was determined for each animal as described in Experiment A. SAP treated animals that did not show selective decrease in ChAT activity within the hippocampus were eliminated from the study.

i. Aversive Stimulus:

Fourteen days following surgery, animals were randomly separated into aversive or non-aversive treatment groups (SAP, 0.22g SAP; aCSF, control aCSF; A, aversive; and NA, non-aversive). The SAP-A and aCSF-A groups 30 min. prior to training, received an injection of sterile saline (10ml/kg, IP), while the SAP-NA and aCSF-NA did not.

3. Results

Of the original 32 animals, two animals (1 aCSF-NA, 1 SAP-NA) failed to run the maze, reach criterion, or displayed side biases and were excluded from the study. Treatment significantly affected the number of days that animals required to reach criterion [$F(3,26) = 6.95$, $p = 0.0014$] (Fig. 15). Specifically, SAP-NA animals required more days to reach criterion than corresponding aCSF-NA controls (21.7 ± 1.6 days versus. 15.9±0.5 days, $p < 0.05$). In contrast, SAP treated animals that also received the mild aversive stimulus did not require significantly more days to reach criterion than corresponding aCSF-A animals (16.8 ± 1.3 for SAP-A animals versus. 13.4 ± 1.4 days for aCSF-A animals, $p > 0.05$). Also, SAP-A treated animals took significantly fewer days to reach criterion than SAP-NA animals ($p < 0.05$), however, there was no significant difference in days-to-criterion between the aCSF-A and the aCSF-NA treated controls ($p > 0.05$). Note that by day 20 of training, all control animals had reached criterion, 7 of 8 SAP-A animals had reached criterion, and only 3 of 7 SAP-NA animals had reached criterion. By day 30 of training, only a single animal in the SAP-A group had not reached criterion.

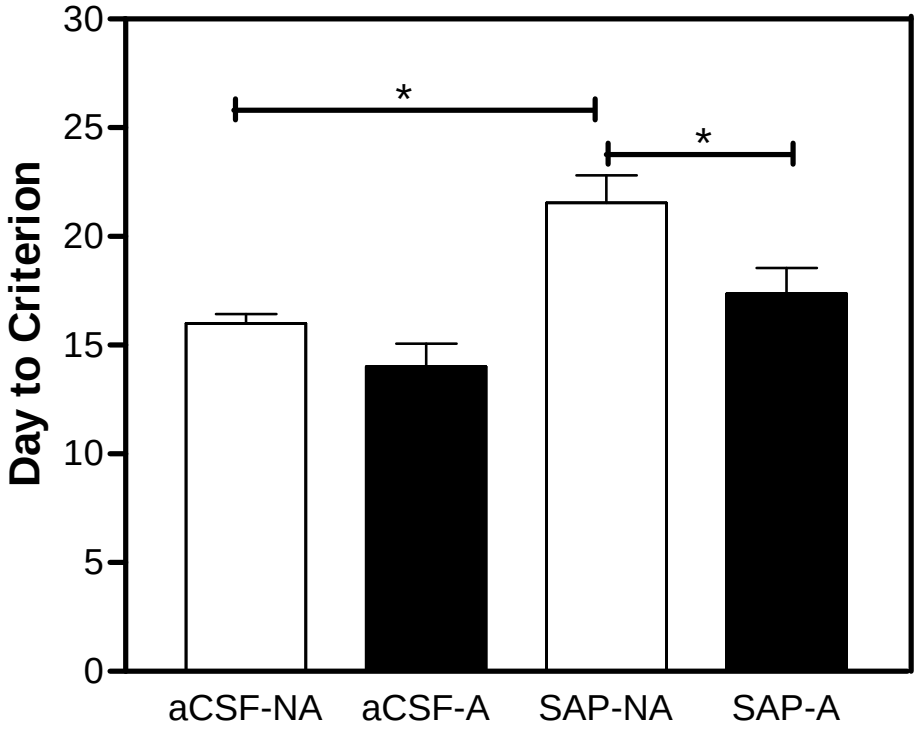


Figure 15. **Effects that the introduction of a mild aversive stimulus had on the number of days to reach criterion in the DMP T-maze task.**

Bar graph representing the effect of an aversive stimulus on the average number of days required to reach criterion for each treatment group. Top of bar represents the mean number of days to reach criterion in the DMP task ± sem. The SAP-NA group required significantly more days to reach criterion than the corresponding aCSF-NA controls. In contrast, the SAP-A group did not require significantly more time to reach criterion than the corresponding aCSF-A controls. * $p < 0.05$.

Analysis of the learning curves (Fig. 16) by two-way ANOVA demonstrated a significant effect of SAP treatment ($F[3,156] = 103.0$ $p < 0.0001$), a significant effect of 'Block' ($F[5,156] = 11.48$, $p < 0.0001$), and a significant Treatment x Block interaction ($F[15,156] = 1.811$, $p = 0.0374$) (Fig. 15). A separate analysis of Blocks 3 and 4 revealed significant differences between treatment groups ($F[3,32] = 3.739$, $p = 0.021$ for Block 3; $F[3,32] = 8.572$, $p = 0.0003$ for Block 4). One-way ANOVA analysis within each block revealed a significant decrease in performance for SAP-NA compared to aCSF-NA treated animals during Blocks 3 and 4. Additionally, the SAP-A group performed significantly better than the SAP-NA group ($p < 0.05$). Differences between the aCSF-A and SAP-A treated groups were not statistically significant during any block.

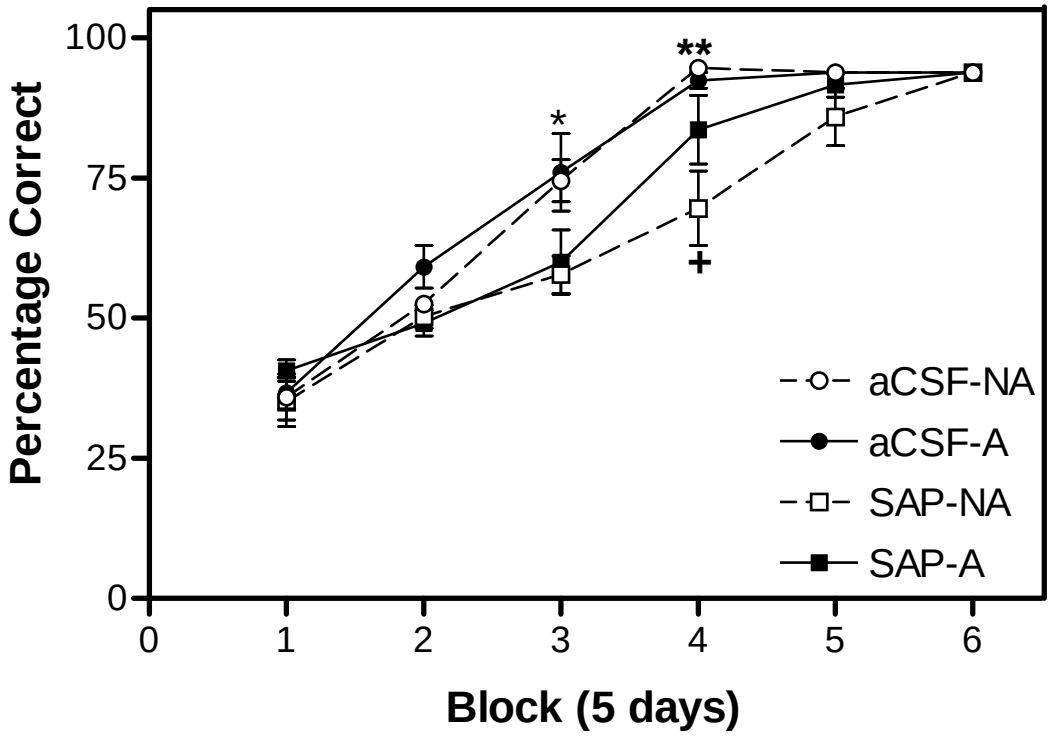


Figure 16: **Effects that the introduction of a mild aversive stimulus had on the rate of acquisition in the DMP T-maze task.**

Learning curves showing acquisition of the DMP task across six 5-day blocks of training. Values represent the mean percentage correct choices for each treatment groups during each period of training. Note that all groups showed improved performance over time; however during Blocks 3 and 4 the performance of the SAP-NA-treated animals was significantly worse than the corresponding aCSF-NA-treated controls (* = $p < 0.05$; ** = $p < 0.01$). Also, note that during Block 4, the performance of the SAP-A-treated animals was significantly better than the SAP-NA-treated animals (+ = $p < 0.05$). Differences in acquisition between the aCSF-Aand SAP-A treatment groups were not statistically significant during any period.

Again we observed that during the early stages of training all of the animals appeared to adopt a turning strategy. Again there was no significant difference in the amount of time that each group took to adopt the turning strategy. The number of days that animals engaged in a turning strategy was significantly increased following SAP lesioning of the medial septum ($p < 0.05$) (Fig. 17). SAP treated animals engaged in a turning strategy for 13.78 ± 1.84 days while aCSF treated controls only engaged in this strategy for 7.75 ± 1.29 days. Interestingly, following the introduction of an aversive stimulus this significant difference was no longer observed (7.89 ± 1.37 for aCSF-A treated animals versus 9.75 ± 1.31 for SAP-A treated animals; $p = 0.54$).

To determine whether the differences seen in days engaged in a turning strategy is enough to explain the effects of treatment differences on acquisition of the DMP task, the days using the turning strategy were subtracted from days required to reach criterion (Fig. 17). Again a one-way ANOVA revealed that once the number of days rats using a turning strategy were subtracted from days to criterion, no significant effect on days to criterion is seen with SAP lesion of the MS ($p = 0.32$) (Fig 18). This indicated that a portion of the effect of the aversive stimulus maybe related to the number of days animals utilized a turning strategy.

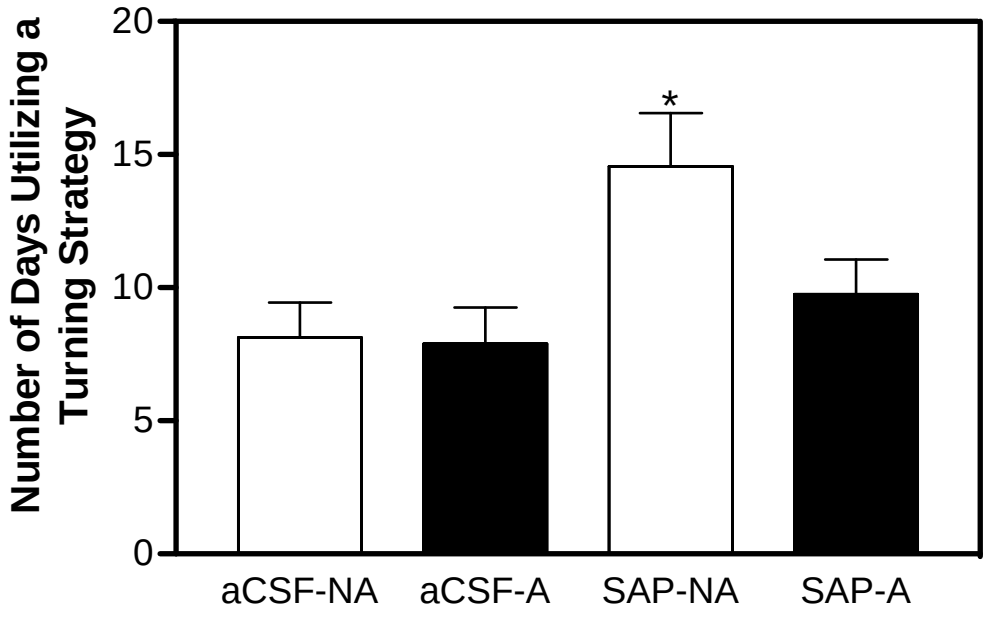


Figure 17: Effects that the introduction of a mild aversive stimulus had on the utilization of a persistent turning strategy.

The number of days rats displayed a turning strategy during DMP T-maze training. The top of the bar represents the mean ± s.e.m. Note that SAP-NA treated animals adopted a turning strategy for more days compared to controls. Also note that there was no significant difference between aCSF-A and SAP-A. *$p < 0.05$ relative to the aCSF-NA treated controls.

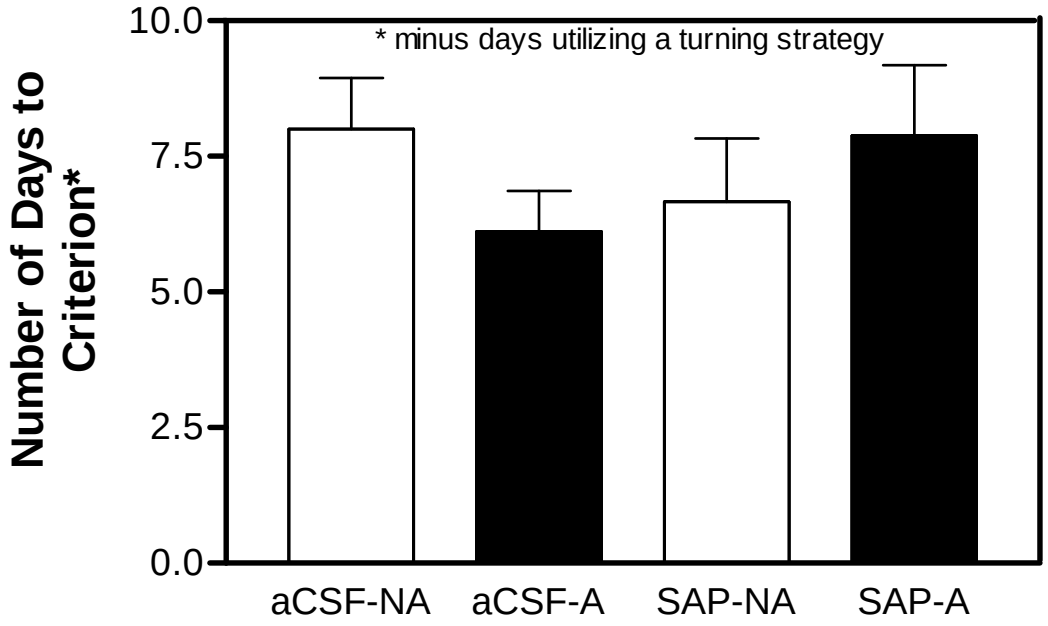


Figure 18: Effect that the introduction of a mild aversive stimulus had on the number of days to reach criterion, excluding the days utilizing a turning strategy.

Days to criterion minus day animals are engaged in a turning strategy. SAP-NA treated animals did not differ significantly in the number of days to reach criterion when compared to controls (*p=0.54*).

4. Discussion

As stated above, the data show that SAP-lesions significantly impaired acquisition of the DMP task relative to controls. As predicted, this effect was significantly reduced by application of a mild aversive stimulus each day prior to training (Fig. 14, 15). This finding demonstrated that a mild aversive stimulus could attenuate or mask a deficit resulting from lesion of MS cholinergic neurons on acquisition of a spatial task. This result is consistent with studies of working memory that used paradigms with an aversive component and found that selective cholinergic lesions of the MS did not produce acquisition deficits in spatial working memory (179-181). Notably, application of the aversive stimulus had no significant effect on DMP acquisition in the non-lesioned control animals. This suggests that the aversive stimulus may only affect performance in the presence of the lesion; however, the possibility of a ceiling effect on performance in the non-lesioned animals cannot be excluded.

One might expect an aversive stimulus to have the greatest effect early during training, when the stimulus is relatively novel, and a lesser effect as the animal becomes habituated to the stimulus later in training. The effect of the aversive stimulus in the SAP-arousal group, though, became apparent relatively late in training (days 16–20; block 4). It is possible that the effect of the aversive stimulus in SAP lesioned animals was the result of the strategy shift that occurred relatively late during acquisition of the task. This may suggest that SAP lesioned rats have diminished ability to habituate to the low level of stree experienced with the i.p. injection. The effect of the i.p. injection may also affect the level of reliance on a single learning strategy for SAP treated animals early in training, which is manifested as diminished rate acquisition later in training. The

SAP treated animals may be more reliant on the turning strategy early in training, decreasing the potential to adopt the more successful strategy. Notably, there was no significant difference in hippocampal ChAT activity between the SAP-A and the SAP-NA treated groups, suggesting that the differences in T-maze acquisition between the two groups was not due to a bias resulting from a greater loss of septo-hippocampal cholinergic projections in one group compared to the other.

The sensitivity of any task to lesions of a single pathway depends on whether the lesion involves the principal pathway activated during the task, as opposed to several pathways that are activated. The hippocampus receives inputs from a variety of cortical and subcortical structures, including the entorhinal cortex, amygdala, medial septum, thalamus, and monoaminergic cell groups in the midbrain and hindbrain. Mild stress has been shown to enhance acquisition of certain spatial learning tasks via hippocampal activation utilizing neuronal pathways that do not involve the MS (164, 217). Therefore, a task with a mildly aversive component may possibly preserve acquisition of a spatial navigation task in animals with a septal-hippocampal cholinergic lesion via pathways that are independent of the septal-hippocampal tract. The result would be a loss of sensitivity with tests for impairments in learning/memory resulting from a septal-hippocampal lesion that involved a mildly aversive stimulus, e.g. the MWM.

One must consider the possibility that there are no purely hippocampal tasks. Most spatial tasks can be solved using a variety of strategies (198), each of which may rely to varying degrees on different neural substrates. As an example, during stress, it is well established that epinephrine released from the adrenal cortex can activate adrenergic

receptors that facilitate memory function (167-169). Adrenergic receptors found on vagal afferents that project to the nucleus of the solitary tract can subsequently activate neurons that project to the amygdala (174), a structure long associated with the acquisition of memories of aversive stimuli (170, 172-174, 213) and the modulation of memory processes involving the hippocampus (175-177). Consequently, it is possible that in addition to enhancing the activity of non-cholinergic inputs to the hippocampus, administration of a mild aversive stimulus could also activate parallel memory systems (219, 220) that enhance performance, perhaps by strengthening the use of strategies less dependent on hippocampal cholinergic inputs. Evidence in support of this theory is that following the introduction of an aversive stimulus, SAP-lesioned rats no longer utilized the persistent turning strategy for a greater number of days than control animals. This result is consistent with, and may help to explain, the reported discrepancies between the effects of septal cholinergic lesions on acquisition of MWM versus land-based (e.g., T-maze) tasks. While both types of tasks can be used to evaluate spatial learning and memory, the levels of stress are higher in a water maze task due to the aversive environment of the pool (221, 222). Therefore, performance of the DMP T-maze task may be more sensitive to SAP-lesion because it is less aversive. This is consistent with the current study that showed administration of a mild stressor eliminated the sensitivity of the DMP task to the cholinergic lesion; thereby, producing results more like those reported with the MWM.

There is a report in which SAP lesion induced a deficit in MWM performance (223). However, the methodology utilized intraventricular injection of SAP, resulting not only in a broader cholinergic lesion of brain structures than intra-parenchymal injection

in the MS, but also the loss of purkinje cells of the cerebellum (224). Other studies, after injecting SAP into the MS and NBM, failed to detect a deficit in performance in the radial arm maze, a non-aversive task (225). However, this result may have been a consequence of the particular environment in which the animals were tested or specific aspects of the testing paradigm.

In summary, the findings demonstrate that cholinergic inputs to the hippocampus play a role in the acquisition of the DMP task under low-stress conditions, but that the impact of the cholinergic lesion is attenuated in the presence of a mild aversive stimulus. In addition, the data suggest caution when interpreting negative findings based on a single behavioral task, or a set of tasks that emulate a single environmental condition.

IV. CONCLUSIONS

The results of these investigations are significant to the ongoing efforts to develop models that mimic memory deficits associated with many forms of dementia as well as developing treatments for these diseases. With Alzheimer's disease, the cholinergic neurons of the basal forebrain are one of the most sensitive targets of the degenerative processes. The results demonstrated that low doses of SAP selectively destroyed cholinergic neurons of the MS in rats; resulting in impaired rate of acquisition of a DMP T-maze task. This provides evidence that SAP-MS lesioned rodents tested in the DMP task can be utilized as a model for cholinergic deterioration seen with aging and certain forms of dementia. Furthermore this model can be used to develop and test new medications which could possibly attenuate the cognitive defects in the DMP task related with diminished cholinergic innervations of the hippocampus. Microdialysis studies using SAP lesioned animals could also explore treatment regiments which are able to increase the cholinergic tone of the hippocampus. Medications which are able to improve learning and memory in SAP lesioned animals may also possibly provide a new treatment for dementia that does not rely on cholinergic actions.

The results also showed that rats during different periods of training in the DMP task adopted different navigation strategies. During the early stages of training the rats used an egocentric (response) strategy manifested as persistent turning; and later switched to an allocentric strategy using extra-maze cues to select the correct goal arm. The diminished rate of acquisition associated with the SAP lesion of the MS was the result of the delayed adoption of an allocentric strategy used to reach criterion. These results add to the growing literature that describes how animals solve different learning

and memory tasks. These results also provide much insight into the function of different neuronal systems during distinct stages of the learning and memory process. In the future, when testing treatments that attenuate the diminished rate of acquisition associated with SAP-MS lesions one could assess the ability of the treatment to alter the utilization of the different learning strategies. To further clarify the neuronal systems involved in the different learning strategies it is vital to develop a microdialysis experiment where changes in the concentrations of different neurotransmitters during the different stages of learning in the DMP task could be determined. It would also be informative to use selective neurotoxins to lesion different neuronal systems and determine how these lesions effect the utilization of learning strategies used in spatial learning and memory tasks.

DHEA and its sulfated ester DHEAS have gained significant interest in the field of neuroscience and DHEA is sold as a herbal remedy for cognitive decline. The exact mechanisms by which DHEAS and steroid sulfatase inhibitors enhance cognition remain unclear. Moreover, the long term safety of these treatments is currently unknown. The results demonstrated that treatments with steroid sulfatase inhibitor, DU-14, further impaired the rate of acquisition of the DMP T-maze task in SAP treated rats. Treatments with differing concentrations of DHEAS were unable to attenuate the impaired rate of acquisition of the DMP T-maze task associated with SAP lesion of the MS. Furthermore, both treatments were unable to improve cognition in the control animals. These results suggest that the memory enhancing effects associated with DU-14 and DHEAS treatments could possible require intact cholinergic afferents to the hippocampus. This may provide a potential mechanism for the memory enhancing and anti-amnestic effects

observed with DHEAS and DU-14 treatments. Before DU-14 can be exclude as a possible treatment for cognitive decline associated with SAP-MS lesions, future studies utilizing different concentrations and multiple learning and memory paradigms need to be performed. Future experiments testing the cognitive enhancing effects of DHEAS should administer the drug through an implanted continue release osmotic pump or at a time which will not interfere with the behavioral testing. In this study and others, DHEAS has been administered as an i.p. injection. When this route of administration is utilized one could not exclude possible peripheral effects. To determine the central effects of DHEAS a study needs to be designed investigating the effects of direct administration of the neurosteriod into the CNS on learning and memory. To better elucidate the mechanism by which DU-14 enhance memory, microdialysis experiments need to be designed that can determine if DU-14 or DHEAS can increase the cholinergic tone of the hippocampus and alter the release of other neurotransmitters in control and SAP-MS treated animals. This could further demonstrate that an intact cholinergic afferent to the hippocampus is need for DU-14 to exhibit its memory enhance and anti-amnestic effects. Since the mechanism by which DHEAS is produced in the brain or enters the CNS is unclear, future studies need to elucidate the mechanism by which DHEAS is produced in the CNS or crosses the BBB. Once these mechanisms are determined new drugs could be developed to target the mechanisms and increase DHEAS levels in the aging and those patients with dementia.

Interestingly, the introduction of a mild aversive stimulus was able to improve acquisition of the DMP task in SAP-lesion rats and decrease the use of a persistent turning strategy. These findings suggest that the activation of functionally parallel

neuronal systems, reduce the influence the hippocampal cholinergic afferents have on the cognitive processes used to acquire the DMP task. When performing behavioral testing one need to take into consideration environmental factors which may enhance the stress and arousal levels of the animals.

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