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Phebian Arinola Abitoye

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THE STEROID SULFATASE INHIBITOR (p-O-SULFAMOYL) –
TETRADECANOYL TYRAMINE (DU-14) ENHANCES MEMORY RETENTION IN
PASSIVE AVOIDANCE IN RATS WITH HIPPOCAMPAL CHOLINERGIC DEFICIT

A Thesis

Presented to the Graduate School of Pharmaceutical Sciences

Duquesne University

In Partial Fulfillment of the requirements for
the degree of Master of Science
(Pharmacology/Toxicology)

By

Phebian Arinola Abitoye

March 10, 2008

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ABSTRACT

THE STEROID SULFATASE INHIBITOR (p-O-SULFAMOYL) –
TETRADECANOYL TYRAMINE (DU-14) ENHANCES MEMORY RETENTION IN
PASSIVE AVOIDANCE IN RATS WITH HIPPOCAMPAL CHOLINERGIC DEFICIT

By

Phebian Arinola Abitoye

May 2008

Thesis Supervised by David A. Johnson, PhD.

Neurosteroids, such as dehydroepiandrosterone sulfate, (DHEAS) are steroidal compounds synthesized within the central nervous system that modulate its function. Among the effects associated with sulfated neurosteroids is the enhancement of memory. Previous studies have demonstrated that altering the metabolism of neurosteroids via inhibition of the enzyme steroid sulfatase could reverse scopolamine-induced amnesia. p-O-(Sulfamoyl) - tetradecanoyl tyramine (DU-14) is a steroid sulfatase inhibitor (SSI) that prevents the conversion of DHEAS to DHEA thereby increasing the concentration of sulfated neurosteroids to potentiate the effect of endogenous DHEAS. To determine whether DU-14 could enhance memory retention for footshock in rats with hippocampal cholinergic hypofunction, male Sprague-Dawley rats were infused with the immunotoxin 192 IgG-saporin (SAP) into the medial septum, and tested using a step-through passive avoidance paradigm. There was a greater than 80% decrease in ChAT activity in the

hippocampi of animals treated with SAP ($p < 0.0001$). There was also a 24.1% decrease in ChAT activity of in the frontal cortex of SAP animals administered DU-14. However, this small but significant decrease did not affect locomotor behavior in rats. Administration of DU-14 enhanced memory retention for 1.0 mA and not 1.25 mA footshock in animals with SAP lesion compared to vehicle treated SAP animals ($p < 0.05$). The effect of DU-14 on locomotor activity was also investigated. DU-14 did not affect locomotor activity during acclimation or retention. These results suggest that DU-14 may enhance memory retention in rats with hippocampal cholinergic lesion.

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LIST OF ABBREVIATIONS

4-mus - 4-methylumbelliferyl sulfate

ACh - Acetylcholine

AChE - Acetylcholinesterase

AD - Alzheimer s disease

ALLO - Allopregnanolone

AMPA - α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

cAMP - Cyclic adenosine monophosphate / 3'-5'-cyclic adenosine monophosphate

CBF - Cholinergic basal forebrain

ChAT - Choline acetyltransferase

CNS - Central Nervous System

CPR - Cardiopulmonary resuscitation

DHA-STS - Dehydroepiandrosterone sulfatase

DHEA - Dehydroepiandrosterone

DHEAS - Dehydroepiandrosterone sulfate

DOC- Deoxycorticosterone

DU-14 - p-O-Sulfamoyl-N-tetradecanoyl tyramine

E1-STS - Estrone sulfatase

EMATE - Estrone 3-O-sulfamate

FX - Frontal Cortex

GABA - γ -aminobutyric acid

GABA-T - GABA transaminase

LIST OF ABBREVIATIONS Cont.

- GAD - Glutamic acid decarboxylase
- GPCR - G-protein coupled receptors
- HIPP - Hippocampus
- IPSP - Inhibitory post synaptic potential
- LNGFR - Low affinity nerve growth factor receptor
- mAChRs - Muscarinic acetylcholine receptors
- MCF-7 - Human breast adenocarcinoma cell line
- MDA-MB-231 - Human Caucasian breast adenocarcinoma
- MS - Medial septum
- ms - Millisecond
- nAChRs - Nicotinic acetylcholine receptors
- NAS - Neuroactive steroid
- nbM - Nucleus Basalis of Meynert
- NMDA - N-methyl-D-aspartic acid
- NS - Neurosteroid
- NSST - Neurosteroid sulfuryl transferase
- P - Progesterone
- p75^{NTR} - Low affinity neurotrophic receptors
- PAPs - Adenosine-3-phosphate-5-phosphosulfate
- Pregs - Pregnenalone
- SAP - 192 IgG- Saporin

LIST OF ABBREVIATIONS Cont.

sec - Seconds

SSD - Succinate semialdehyde dehydrogenase

SSI - Steroid sulfatase inhibitor

STS - Steroid sulfatase/Estrone sulfatase/arylsulfatase C

VGAT - Vesicular inhibitory amino acid transporter

CSF – Cerebrospinal fluid

Co – Control

I. INTRODUCTION

A. Statement of Problem

Alzheimer's disease, the most common form of senile dementia, is a progressive neurodegenerative disorder characterized by memory loss and behavioral changes. It is a major killer diseases ranking fourth or fifth in the United States. Alzheimer's disease affects memory functions predominantly related to the hippocampus and the cerebral cortex and is associated with a significant loss of cholinergic neurons. This disease is projected to increase in prevalence during the coming decades, as the life span of United States population gets longer. With more drugs becoming available, it may become possible to delay disease progression for many years [1]. A few partially successful therapies for the treatment and /or prevention of the disorder include augmentation of brain cholinergic function by inhibitors of acetylcholinesterase (AChE), e.g., physostigimine, tacrine and donepezil. An alternative strategy is the use of NMDA glutamate receptor antagonist memantine [2]. Another approach currently being investigated is the modulation of the major inhibitory GABA neurons. Neurosteroids have been implicated in memory enhancement over the past decade [3, 4] and further research has shown that preventing the conversion of these steroids, especially DHEAS, to the unsulfated form can enhance memory function [5]. The goal of this project is to determine whether chronic administration of a steroid sulfatase inhibitor, DU-14, can enhance memory retention in rats with a saporin induced selective cholinergic deficit.

B. Literature Review

1. Learning and Memory

Learning is the acquisition and retention of memories and behaviors including skills, knowledge, understanding, values and wisdom. It is the goal of education and the product of experience. It can also be said to be a relatively permanent process that is inferred from performance changes due to practice. Thus skills in reading, which obviously result from practice, are instances of learning. Similarly, improvement in athletic skills such as golf or tennis are instances of learning because they involve changes which can be attributed to a practice condition [6]. The current understanding of neurons and central nervous system (CNS) function implies that the process of learning corresponds to changes in the relationship between certain neurons in the brain. Generally however, it is recognized that the retention of memories comes easier when parts of the brain that control multiple functions such as hearing, seeing, smelling, motor skills, touch sense, the application of informal names and reason are stimulated.

Memory on the other hand can be defined as a behavioral change caused by a previous experience [7]. Memory is a fundamental process, and without memory, there is a capability of nothing but simple reflexes and stereotyped behaviors. Thus, learning and memory are one of the most intensively studied subjects in the field of neuroscience [7]. Learning and memory are two distinct, yet intimately associated, processes. Quite simply, without memory, there is no learning [8]. In psychology, memory is an organism's ability to store, retain, and subsequently recall information. Traditional studies of memory began in the realm of philosophy, including techniques of artificially

enhancing the memory. The late nineteenth and early twentieth century put memory within the realm of cognitive psychology.

Memory classification

There are several ways to classify memories, based on duration, nature, and retrieval of information.

Classification based on processing

From an information processing perspective, there are three main stages in the formation and retrieval of memory:

- Encoding or registration (processing and combining of received information)
- Storage (creation of a record of the encoded information)
- Retrieval or recall (calling back the stored information in response to some cue for use in a process or activity)

Classification based on duration

A basic and generally accepted classification of memory is based on the duration of memory retention. In this classification system there are four distinct types of memory: sensory memory, short-term memory, intermediate memory and long-term memory.

Sensory memory

This can also be referred to as immediate memory [9]. Sensory memory corresponds approximately to the initial 200-500ms after an item is perceived. The

ability to look at an item, and remember what it looked like with just a second of observation is an example of sensory memory. With very short presentations, participants often report that they seem to “see” more than they can actually report. This type of memory cannot be prolonged via rehearsal.

Short-term Memory

Some of the information in sensory memory is then transferred to short-term memory. Short-term memory allows one to retain something from several seconds to as long as a minute without rehearsal. Its capacity is also very limited. A conventional way of testing the integrity of short-term memory is to present a string of randomly ordered digits, which the individual is then asked to repeat [9]. A special sort of short-term memory is called working memory. This refers to the ability to hold information in mind long enough to carry out sequential actions [10]. An example is searching for a lost object; working memory allows the hunt to proceed efficiently, avoiding places already inspected. A particular advantage to this type of memory is that it can be readily examined in experimental animals [9].

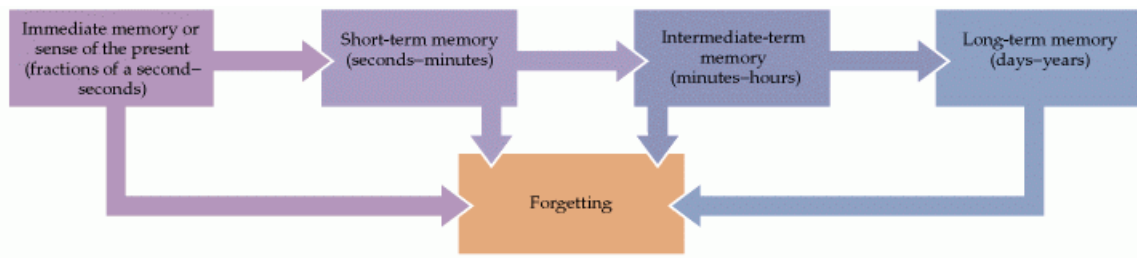


Figure 1: The major temporal categories of human memory [9]

Intermediate memory

This type of memory occurs once short-term memory has been processed. It lasts from minutes to hours. It is the part of memory which holds and mixes information from the different parts of memory.

Long-term memory

The storage in sensory, short-term and intermediate memories generally has a strictly limited capacity and duration, meaning that information is available over a certain period of time, but is not retained indefinitely. By contrast, long-term memory can store much larger quantities of information for potentially unlimited duration (sometimes a whole life span). Short-term memory is supported by transient patterns of neuronal communication, dependent on regions of the frontal lobe (especially dorsolateral prefrontal cortex) and parietal lobe. Long-term memories, on the other hand, are maintained by more stable and permanent changes in neural connections widely spread through the brain.

The most popular candidate site for memory storage is the synapse or set of synapses, where nerve cells (neurons) communicate. A change in transmission efficacy at the synapse has been considered to be the mechanism of memory [7]. It is widely accepted that protein synthesis, including local synthesis at synapses, enables the control of synaptic strength independent of cell body via rapid protein production from pre-existing mRNA, and this is required for several forms of synaptic plasticity [11-13]. This is rooted in the fact that synaptic plasticity is a physiological phenomenon whereby

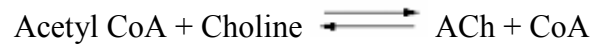
specific patterns of neural activity give rise to changes in synaptic efficacy and neural excitability that long outlast the event that trigger them [14]. Memory entails a number of biological strategies and anatomical substrates that are unlikely to be explained in terms of any particular cellular or molecular mechanism. Various neurotransmitters in the brain, including cholinergic, γ -aminobutyric acid, adrenergic and glutamatergic systems have been shown to play a significant role in memory function [15-17].

2. The Cholinergic System

The cholinergic system is one of the most important modulatory neurotransmitter systems in the brain [2, 18], and has long been recognized as playing a key role in the regulation of cognitive functions [2, 17, 19, 20]. Briefly, the cholinergic system is distributed in a variety of different nuclei of which two groups (basal forebrain and pedunculopontine) have extensive projections to the cortex and thalamus. Pedunculopontine cholinergic neurons are considered to control rapid eye movement sleep or dreaming while basal forebrain cholinergic pathways appear to generate and integrate consciousness and awareness [18]. The cholinergic system is involved in higher brain functions, including learning and memory. The hippocampal region of the brain is extensively innervated by cholinergic fibers originating mainly from the septal nucleus, and the vertical limb of the diagonal band of Broca [21, 22].

The role of acetylcholine in memory has been well established. The neurophysiological activity of ACh has been known since the turn of the century and its neurotransmitter role since the mid-1920s [23]. Acetylcholine is synthesized in a

reaction catalyzed by choline acetyltransferase (ChAT) and broken down by another enzyme called acetylcholinesterase (AChE).



The acetyl CoA for ACh synthesis arises from glucose through glycolysis and the pyruvate dehydrogenase system [23, 24]. Choline on the other hand can be obtained either from the hydrolysis of acetylcholine or breakdown of phosphatidylcholine, a membrane phospholipid.

Cholinergic pathways in the central nervous system (CNS)

The identification of cholinergic synapses in the peripheral nervous system was relatively easy, and it has been known that acetylcholine is the transmitter at autonomic ganglia, at the parasympathetic postganglionic synapses and the neuromuscular junction [2, 23]. In the CNS, cholinergic neurons exhibit two basic organization schemata: local circuit cells and projection neurons. Local circuit cells (i.e. those that are morphologically arrayed wholly within the neural structure in which they are found) are exemplified by the interneurons of the caudate-putamen nucleus, nucleus accumbens, olfactory tubercle and Islands of Calleja complex. Projection neurons connect two or more different regions. Of the cholinergic projection neurons that interconnect central structures, two major subconstellations have been identified. The first is the basal forebrain cholinergic complex composed of ChAT-positive neurons in the medial septal nucleus, diagonal band nuclei, substantial innominata, magnocellular preoptic field, and

nucleus basalis, all of which project to the entire nonstriatal telencephalon. The second is the pontomesencephalotegmental cholinergic complex composed of ChAT-immunoreactive cells in the pedunculo-pontine and laterodorsal tegmental nuclei, which projects ascendingly to the thalamus and other diencephalic loci and descendingly to the pontine and medullary reticular formations, deep cerebellar and vestibular nuclei, and cranial nerve nuclei [23].

Cholinergic receptors

Cholinergic receptors fall into two classes, muscarinic and nicotinic. In mammals, five distinct subtypes of muscarinic ACh receptors (mAChRs) have been identified, each produced by a different gene. These variants have distinct anatomical locations in the periphery and CNS and differing chemical specificities. They are G protein-coupled receptors (GPCRs) linked to a variety of second messenger systems. When activated, they act either directly on ion channels or are linked to a variety of second messenger system. The final effect can be to open or close potassium, calcium or chloride channels leading to either a depolarization or a hyperpolarization of neurons. M₁, M₃, and M₅ are coupled to phosphatidyl inositol hydrolysis while M₂ and M₄ are coupled to cAMP. The nicotinic ACh receptors (nAChRs) are members of a superfamily of ligand gated ion channels. Each receptor is made up of five receptor subunits arranged symmetrically around a central pore structure whose activation always causes a rapid increase in cellular permeability to sodium and calcium, depolarization and excitation. The receptors exist at the skeletal neuromuscular junction, autonomic ganglia, adrenal medulla and in the CNS [2, 23].

The cholinergic system and memory

Both muscarinic and nicotinic cholinergic receptors have been shown to be involved in cognitive function [25]. However, the action of ACh on learning and memory is mostly mediated by mAChRs [22]. Cholinergic transmission at the mAChRs has been implicated in learning and memory in humans and other mammals and also shown to be important in the active maintenance of short-term memories and hippocampal plasticity [10, 25, 26].

The role of muscarinic receptors in memory function is evident in studies of M₂ muscarinic acetylcholine receptor knock out mice that show deficits in behavioral flexibility, working memory and hippocampal plasticity [26]. The experiments included two different hippocampus-dependent spatial learning and memory tasks; the Barnes circular maze test and the T-maze delayed alternation task. Significant deficits in behavioral flexibility and working memory were demonstrated to be associated with pronounced impairments in neuronal plasticity at the Schaffer collateral-CA1 synapse in M₂^{-/-} mice. In summary, the study found that M₂ receptors are involved in processes that facilitate working memory.

Drugs that elevate, mimic, or inhibit the breakdown of ACh improve learning and memory performance [25]. Cholinergic innervations of the cerebral cortex and the hippocampus originate primarily from the cholinergic basal nuclear complex. Lesions of these basal forebrain cholinergic neurons have been reported to result in impairment in memory, learning, and attention, whereas cholinergic agonists facilitate learning and memory [27]. Conversely, drugs that deplete ACh or compete for its receptor sites reduce performance of memory tasks.

Dementia of the Alzheimer's type involves loss of ACh activity, and cellular degeneration in the nucleus basalis of Meynert (nbM) and the cortex. Nicotinic cholinergic neurons are especially depleted [25]. The degeneration of forebrain cholinergic projections is one of the most salient neurochemical features of Alzheimer's disease AD, although other transmitter systems are also altered in some, but not all, patients.

Cholinergic based therapies of AD have generally failed thus far, or resulted in limited clinical improvements [28]. To date, attempts to treat progressive dementia with cholinergic replacement have been generally disappointing. The hope was that facilitation of ACh function might be therapeutic in a fashion analogous to levodopa treatment in Parkinson's disease, but most studies have reported only modest reduction of dementia following ACh therapy. More recently, drugs such as the AChE inhibitor donepezil (a cerebroselective and reversible anti-AChE) have targeted the cholinergic system for treatment of the memory loss associated with Alzheimer's disease [25, 28, 29]. Another approach is based on the use of precursors of acetylcholine (e.g. lecithin or Choline). Functional data from various laboratories now suggest that the drugs used in clinical trials may not have been ideal, since they can result in the activation of presynaptically located negative M₂-muscarinic autoreceptors either by increasing the half-life of ACh or by direct stimulation by agonists that would inhibit the subsequent release of ACh from remaining functional synapses. Moreover, it has recently been demonstrated that the normal processing of the β -amyloid precursor is influenced by cholinergic activity [28]. Hence, altered cholinergic innervation could lead to an aberrant processing of β -amyloid and possible formation of neurotoxic fragments leading

ultimately to neuritic plaque formation. In addition, in the advanced stage of this progressive form of dementia, extensive loss of cholinergic neurons could prevent individuals from being responsive to therapeutic agents that would target those sites.

Whereas most published research has focused on the importance of septohippocampal cholinergic mechanisms in learning and memory and in generation of the theta rhythm (a wave pattern electroencephalogram in the frequency band of 4-7Hz) [30], the role of septohippocampal GABAergic neurons is increasingly recognized, especially with regard to production of theta activity. Wu et al. [21] suggested that activation of septohippocampal GABA neurons, and not septohippocampal cholinergic neurons, may underlie the behavioral and electrophysiological effects observed after intraseptal infusions of muscarinic agonists in rats.

3. The GABAergic system and memory

The amino acid γ -aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian CNS [2]. GABA was first identified as a unique chemical constituent of the brain in 1950, but its potency as a CNS depressant was not immediately recognized. GABA was initially identified as the only inhibitory amino acid found exclusively in nerves that inhibit the crustacean stretch receptor; moreover, the GABA content accounted for the inhibitory potency of extracts from these nerves. Finally, GABA release correlated with the frequency of nerve stimulation, and identical increases in muscle chloride conductance accompanied GABA application and stimulation of the inhibitory nerve. These same physiological and pharmacological properties helped to establish a role for GABA in the mammalian CNS [31, 32]. There is

substantial data to support the idea that GABA is found primarily in inhibitory interneurons impinging on and modulating activity in the vertebrate brain and plays an important role in information processing. GABA is thought to regulate the balance of excitatory and inhibitory states in the cortex and hippocampus and GABA receptor agonists and antagonists have been variously demonstrated to enhance or inhibit memory processing [31].

GABA receptors have been divided into three main types: A, B and C. The most prominent GABA_A-receptor subtype is a ligand-gated chloride ion channel, an ionotropic receptor that is opened after release of GABA from presynaptic neurons. The GABA_B receptor is a G protein-coupled receptor (GPCR), while the GABA_C receptor is a transmitter-gated chloride channel [2].

GABA_A receptors are the major inhibitory neurotransmitter receptors in the brain and the site of action of many clinically important drugs [32]. Synaptically-released GABA acts at the GABA_A ligand-gated ion channel present on the postsynaptic membrane to produce membrane hyperpolarization through an increase in chloride conductance that comprises fast inhibitory post synaptic potentials, IPSPs [31]. This decreases the depolarizing effect of an excitatory input, thus depressing excitability [32]. GABA_A channels typically consist of two α , two β and one γ subunit. GABA_A is present throughout the mammalian CNS and is the primary effector of GABA-mediated IPSPs.

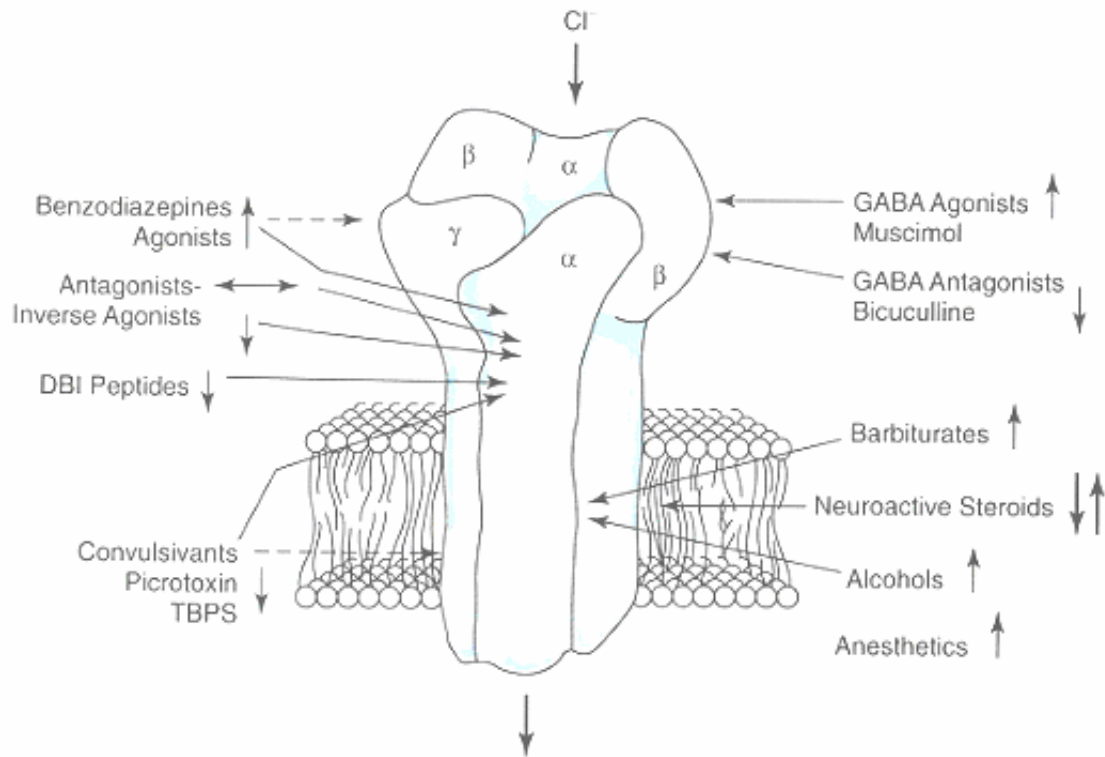


Figure 2: Schematic illustration of GABA_A receptors containing two α and β subunits and a single γ subunit.

GABA is an agonist to the GABA channel receptor but not the only molecule that can modify this channel receptor's opening. Other molecules can also affect it, such as the benzodiazepines, barbiturates, picrotoxin and steroids. By binding to this receptor at different sites from GABA, these molecules modulate the receptor by causing either an opening (e.g. benzodiazepines) or closing (e.g. steroid) of the chloride ion channel [32].

Neuroactive steroids such as estradiol, progesterone, and progesterone's metabolite- allopregnanolone (ALLO) modulate the function of neurotransmitters implicated in regulation, cognition, and behavior. Estradiol enhances neuronal excitability in structures such as the hippocampus via modulation of glutamatergic NMDA receptors. It also has antagonist activity at the GABA_A receptor by reducing the GABA-mediated inhibition. In contrast, the progesterone metabolite ALLO is a potent

GABA_A receptor agonist with anticonvulsant, hypnotic, and anxiolytic effects. As the primary inhibitory neurotransmitter, GABA is released by neurons throughout the brain and has numerous interactions with other neurotransmitter systems. Thus, substances which modulate GABA neurotransmission will not only alter the balance in neuronal excitation and inhibition but are likely to alter the activity of neurons from multiple systems [33]. That the GABA_A receptor, is sensitive to physiologic changes in estradiol and ALLO levels suggests that this area would be fertile ground for research in memory function.

4. Neurosteroids

The term “neurosteroid” (NS), defined as steroids produced within the nervous system, was first introduced by Baulieu in 1981 [30]. The neurosteroid dehydroepiandrosterone sulfate (DHEAS) was found at high levels in the brain long after gonadectomy and adrenalectomy, and was later shown to be synthesized by the brain [34]. Later, androstenedione, pregnenolone, their sulfates and lipid derivatives, as well as tetrahydrometabolites of progesterone (P) and deoxycorticosterone (DOC) were identified as neurosteroids.

There are differences in synthesis pathways for steroids originating in the brain and in the adrenals [34]. In the adrenals, the enzymes responsible for synthesis are never expressed in the same cell [34, 35]. In the brain, the enzymes are co-expressed not only in the same region, but even within the same cell. Contrary to neurosteroids, neuroactive steroids (NAS) refer to steroids that, independent of their origin, are capable of modifying neural activities. NAS bind and modulate different types of membrane

receptors. Neuroactive steroids play a key role in both normal physiology and in the pathogenesis of brain diseases [36]. In spite of poor permeability through the blood–brain barrier for sulfated steroids, high concentrations of DHEA and also its sulfate have been found in brain tissues [36]. Physiological concentrations of dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) are maintained by two enzymes present in the blood and other peripheral tissues, as well as in the brain, steroid sulfatase and neurosteroid sulfuryl transferase. Neurosteroids possess both rapid, non-genomic effects, as well slow genomic effects. In contrast to steroids synthesized in classic steroidogenic tissues that activate cytoplasmic receptors, neurosteroids predominantly use signaling pathways common for neuromodulators. It is well known that unconjugated forms of neurosteroids, such as saturated progesterone metabolites 5 α -pregnan-3 α -ol-20-one (allopregnanolone), its 21-hydroxylated metabolite, and also androsterone and DHEA act on GABA_A receptors as positive modulators that along with other events increase the permeability of chloride ion channels. Sulfated forms of neurosteroids act in the opposite way, as negative GABA_AR modulators. Some neurosteroids conjugated with sulfuric acid, including DHEAS and pregnenolone sulfate (PregS), are also potent positive noncompetitive modulators of NMDA receptors. Δ^5 -Ene-3-hydroxy steroids accumulate in the brain in the form of conjugates, namely, fatty acids esters or sulfates, and their formation has been found to be independent of neurosteroid biosynthesis in the peripheral endocrine tissues [36]. The typical route of biosynthesis *in situ* is the esterification of a free steroid molecule by neurosteroid sulfuryl transferase (NSST). Two enzymes maintain the equilibrium between PregS and Preg or DHEAS and DHEA:

steroid sulfatase (EC 3.1.6.2), which catalyzes unconjugated steroid formation and the already mentioned NSST, which catalyzes steroid sulfate formation.

Neurosteroid sulfuryl transferase is a cytosolic enzyme, which catalyzes the sulfoconjugation of 3 β - or 17 β -hydroxysteroids [36]. It utilizes adenosine-3-phosphate-5-phosphosulfate (PAPS) as a co-substrate, which is formed by a common metabolic pathway, regardless of the species or tissue of origin. The sulfo-conjugation of steroids involves the transfer of a sulfonate radical (SO₃) from an activated donor molecule (PAPS) to a hydroxyl acceptor site. Steroid sulfatase is bound to the membrane of the endoplasmic reticulum, presumably through multiple transmembrane and other membrane anchoring segments. The highest sulfatase activity, therefore, is found mainly in the microsomal fraction. Although steroid sulfatases and sulfotransferases from various tissues have been well characterized, especially estrone sulfatase in connection with breast cancer, there is not enough information about both enzymes in the primate brain, despite the fact that they play important roles in brain physiology and pathophysiology.

Quantitatively, the adrenal DHEA and its sulfate ester DHEAS are the most abundant C₁₉ steroids circulating in human plasma. Even though the plasma concentration of DHEAS decreases with aging, there is still about 1 μ M of this compound in men and women of advanced age [37].

Neuroactive steroids bind to and modulate different types of membrane receptors. The GABA_A and sigma receptor complexes have been the most extensively studied although glycine-activated chloride channels, nicotinic acetylcholine receptors (constituted in *Xenopus laevis* oocytes), mouse striatal and thalamic synaptosomes, and

voltage-activated calcium channels are also modulated by NAS. Within the glutamate receptor family, NMDA receptors, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and kainate receptors have also been demonstrated to be a target for steroid modulation [34].

Among the effects associated with neurosteroids is the enhancement of memory. The mechanism for this enhancement remains unclear; however, DHEAS has been shown to reverse scopolamine-induced amnesia [38]. GABAergic agents in the medial septal nucleus modulate the activation of the hippocampus and ACh turnover. In addition, the mechanism for reversal of scopolamine-induced amnesia has been associated with increased release of ACh [39].

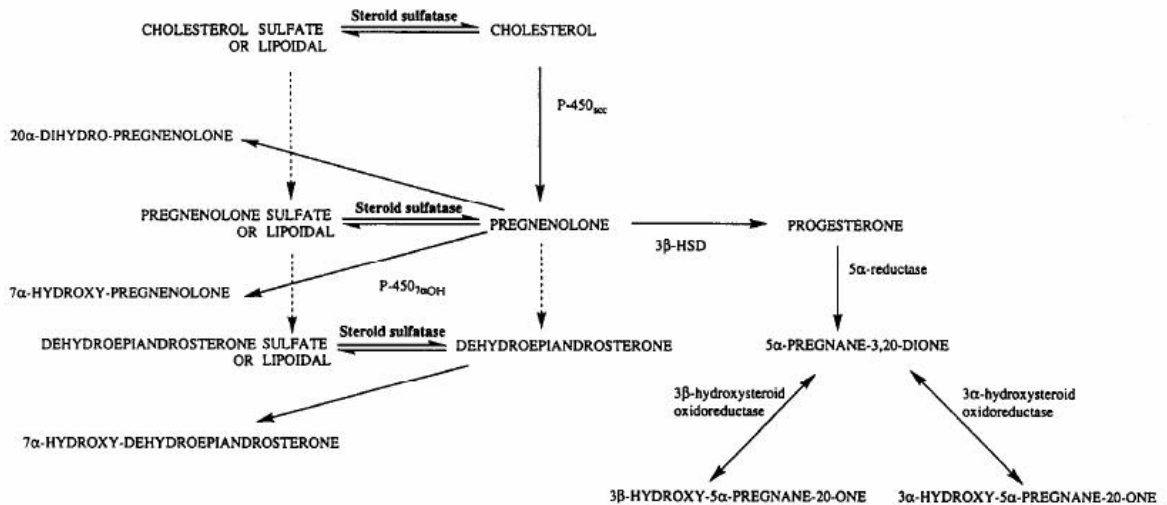


Figure 3: Neurosteroid biosynthesis and metabolism in the rat brain. Dotted arrows indicate metabolic conversions not yet formally demonstrated [5].

5. Steroid sulfatase enzyme

Steroid sulfatase (estrone sulfatase, arylsulfatase C, (STS); E.C. 3.1.6.2) is a ubiquitous membrane-bound, microsomal enzyme localized mainly in the endoplasmic reticulum and the nuclear envelope of cells. It is an enzyme involved in the local production of estrogens and androgens in target organs [40, 41]. It is responsible for the hydrolysis of 3 β -hydroxy steroid sulfates, such as cholesterol and pregnenolone sulfate, and has an important role in regulating the synthesis of estrogenic steroids from estrone sulfate and dehydroepiandrosterone sulfate in hormone-dependent tumors [42]. Preliminary results indicate the importance of estrone sulfate as a potential source of estrogen to support the growth of estrogen-dependent breast cancer [43]. In the skin, the abundant systemic precursor DHEAS is cleaved by steroid sulfatase to DHEA, which is then converted to the bioactive androgens testosterone and dihydrotestosterone. Steroid sulfatase inhibition has been proposed as a novel therapeutic concept for the treatment of androgen-dependent disorders of the hair follicle, such as androgenic alopecia and hirsutism, in addition to acne.

In the brain, DHEAS is cleaved to DHEA via STS, and inhibition of STS enhances learning and spatial memory in rats [44]. Thus, potent inhibitors of estrone sulfatase are potential therapeutic agents for treatment of memory loss. These results suggest a possible role for sulfatase enzyme inhibitors in treating Alzheimer's disease.

A variety of different reversible and irreversible inhibitors of STS have been developed. The most potent and most commonly used inhibitors are steroidal or nonsteroidal *O*-sulfamates, which irreversibly inactivate the enzyme. Sulfatase inhibition was measured using placental microsomes, lysates of cells transfected with an STS

expression vector, or highly purified STS obtained from a cell line stably expressing human STS. Radioactively labeled estrone sulfate or DHEAS and a fluorogenic compound, 4-methylumbelliferyl sulfate (4-MUS), are most commonly used as substrates for the determination of enzyme activity [40, 41].

6. p-O-Sulfamoyl-N-tetradecanoyl tyramine (DU-14)

A number of steroid sulfatase inhibitors have been developed including estrone 3-*O*-sulfamate (EMATE) (**1**), estrone 3-*O*-methylthiophosphonate (**2**), and estrone 3-sulfonyl chloride (**3**). EMATE was the first steroid sulfatase inhibitor exhibiting potent active site-directed irreversible inhibition against both E1-STS and DHA-STs, both *in vitro* and *in vivo* orally or subcutaneously [41]. EMATE was synthesized by treating the sodium salt of estrone with sulfamoyl chloride. This compound inhibited not only estrone sulfatase but also dehydroepiandrosterone sulfatase activity in placental microsomes and in intact MCF-7 breast cancers [42]. Unexpectedly, however, EMATE was found to be a potent estrogen, being five times more active than ethinylestradiol when administered orally to rats [41]. The exact reasons why EMATE is such a potent estrogen remain to be elucidated. However, current evidence suggests that it is acting as a prodrug for estrone.

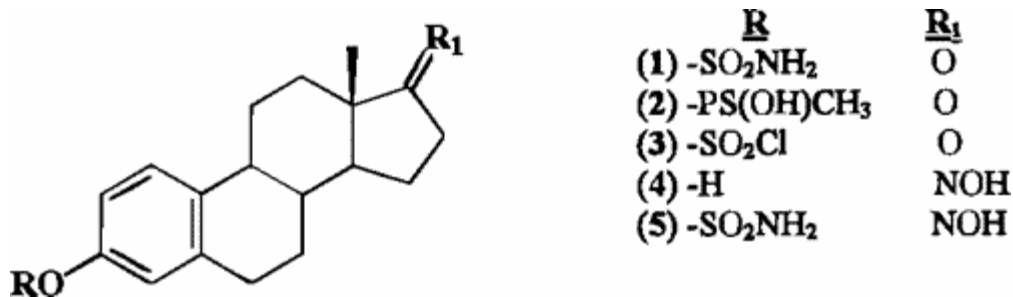


Figure 4: Structures of steroid sulfatase inhibitors (1-3, 5) and estrone oxime (4)

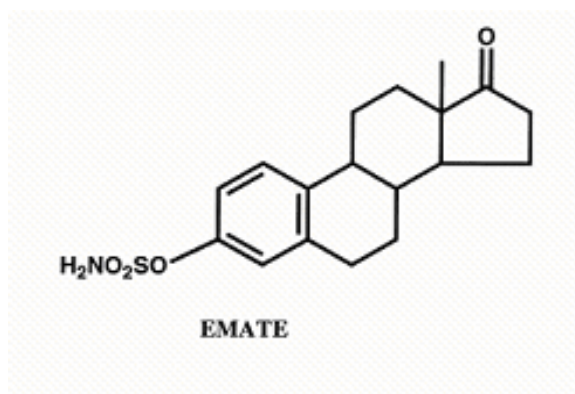


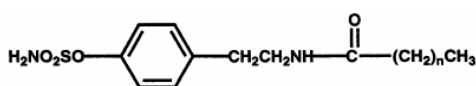
Figure 5: Structure of EMATE, (*p*-*O*-sulfamoyl)-*N*-tetradecanoyl tyramine (C₂-14) [45].

EMATE (**1**) is one of the most potent steroidal E1-STS inhibitors yet synthesized. *In vitro*, EMATE inhibits E1-STS activity by greater than 99% at 0.1 μM in intact MCF-7 breast cancer cells and in a time- and concentration-dependent manner in a placental microsomes preparation, indicating that it acts as an irreversible inhibitor. Subsequent studies have also shown that EMATE inhibits DHA-STS, the enzyme that regulates the biosynthesis of the estrogenic steroid androstenediol [41].

To avoid the problems associated with a potentially active steroid nucleus, a series of (*p*-*O*-sulfamoyl)-*N*-alkanoyltyramines as nonsteroidal estrone sulfatase inhibitors were synthesized and tested [46]. The nine compounds differed in the length of their alkanoyl chains. The ability of the (*p*-*O*-sulfamoyl)-*N*-alkanoyl tyramines to inhibit: (a) estrone sulfatase activity in intact cultures of human breast cancer cells (MDA-MB-231); and (b) the growth of estrogen-dependent human breast cancer cells (MCF-7) were tested. All of the test compounds (1 μM) inhibited the estrone sulfatase activity of intact MDA-MB-231 cells; however, compounds with a longer alkanoyl chain were more effective than those with a shorter chain. The two most potent compounds

were found to be (p-O-sulfamoyl)-N-tridecanoyl tyramine and (p-O-sulfamoyl)-N-tetradecanoyl tyramine (DU-14). It was shown that the (p-O-sulfamoyl)-N-alkanoyl tyramine analogues are also effective inhibitors of estrone sulfatase activity in human breast cancer cells. It was also shown that both C₁₄ and estrone-3-sulfamate are irreversible inhibitors of the estrone sulfatase enzyme [46].

A



Compound	n
C7	5
C8	6
C9	7
C10	8
C11	9
C12	10
C13	11
C14	12
C15	13

B

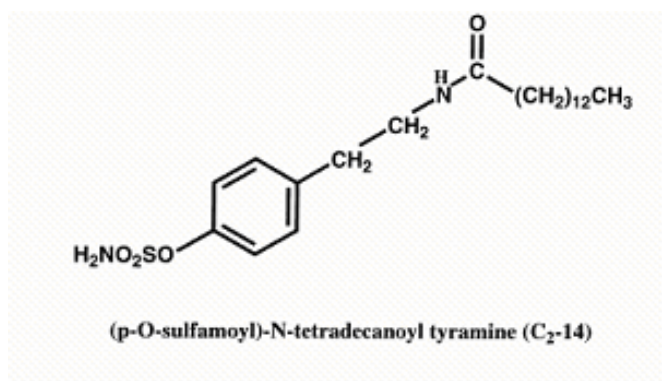


Figure 6: A: Chemical structure of (p-O-sulfamoyl)-N-alkanoyl tyramines B: (p-O-sulfamoyl)-N-tetradecanoyl tyramine (C₂-14 also known as DU-14)

7. Passive/ Inhibitory Avoidance Behavior and Memory

Passive avoidance learning is a generic term for learning to avoid aversive stimuli by "not taking a certain action". The paradigm requires the subject not to initiate a specific type of behavior, usually escape or avoidance, in order to preclude the administration of an aversive event [47]. The most common paradigm is passive avoidance learning of the step-through type. Inhibitory avoidance, also frequently referred to as passive avoidance, requires that subjects (typically rodents) behave in a manner contrary to their normal inclination or predilection. In most cases, subjects must act if they are to avoid the consequences of a negative reinforcer. During the training component of the task, the animal is punished for making an instinctive response, such as moving from a brightly lit chamber into a darkened one (a highly probable event for rodents). Passive avoidance learning is believed to be based on contextual memory, which is associated with the place and the event of "being given the electric shock in the dark box". The hippocampus plays an important role in contextual memory; injuries of the hippocampus decrease the performance of passive avoidance learning [47]. Passive avoidance learning involves not only contextual memory but also amygdala-dependent emotional memory, "fear of the dark box". The performance of passive avoidance learning decreases with defects in either contextual memory or emotional memory.

Inhibitory avoidance behavior and memory assessment.

This test uses an apparatus with a lighted box and a connected darkened box. Because mice/rats prefer a dark place, they naturally move into the dark box when placed in the lighted box. However, when a mouse/rat is given a footshock through the grid

floor of the dark box, the animal hesitates to move into the dark box on a subsequent trial. The intensity of the shock is the minimum sufficient to cause flinch and vocalization [48]. The reason why the mouse/rat hesitates to move into the dark box, which they inherently prefer, is due to learning and memory. The test of passive avoidance learning assesses the ability of a mouse/rat to learn and remember the aversive stimulus on the basis of time, that is; delay latency before entering the dark box.



Figure 7: The Passive Avoidance Apparatus.

The measurement of the latency (retention test) is usually performed after 24 hours or more following the administration of the electric footshock. A prolonged time between an electric shock and the test allows memories of a longer duration to be examined. Presumably the longer the avoidance latency, the more efficacious the memory process [47]. Traditionally, passive avoidance tasks have been used to evaluate

learning and behavioral manipulations. This type of learning is based on the assumption that a biologically probable response terminated by an unpleasant event will be inhibited in the future. The resulting increase in response latency reflects the strength of the memory trace [49].

Potential drawbacks of the inhibitory avoidance paradigms include an often large inter-subject variability, and the differing tolerance to shock reported among subjects (which is probably one reason for the large number of subjects often required when detecting modest changes in task performance). Another disadvantage of the passive avoidance paradigm, at least with respect to other types of delayed response tasks that use changeable discriminable stimuli, is that in inhibitory avoidance, a subject can only be used for a single trial, and thus cannot serve as its own control. However, inhibitory avoidance is a classic paradigm that is well studied. The Medline database (1996-1999) listed over 600 articles with passive avoidance or inhibitory avoidance as a main subject [47]. Thus, in addition to its relative longevity, the method continues to be used extensively. As an example, it was used to substantiate the findings in the quantification of hippocampal and caudaoptamen injury 3 or 7 days after cardiopulmonary resuscitation (CPR) [47, 49, 50].

8. SAP Lesioning

Saporin is a ribosome-inactivating protein, molecular weight 30kD, found in the seeds of the plant *Saponaria officinalis* [51]. 192 IgG-Saporin (SAP) is a chemical conjugate of a mouse monoclonal antibody to rat low affinity neurotrophic receptors

(p75^{NTR}) and the ribosome-inactivating protein [52]. SAP has a molecular weight of 210kD. Intraventricular injection of SAP in almost complete elimination of LNGFR (p75^{NTR})-positive cells in rat [53, 54]. SAP is directed to a cell-surface p75^{NTR} that is selectively expressed at high levels on neurons of the cholinergic basal forebrain (CBF). The p75^{NTR}, is not expressed on the neighboring, non-cholinergic neurons; thus its selectivity. The immunotoxin provides researchers with a powerful tool for neuronal lesioning that is more specific and effective than chemical, surgical or electrolytic lesioning. Various studies have demonstrated both selectivity and efficacy for SAP [54-56].

Elimination of Specific Cell Type

- SAP recognizes p75^{NTR}-expressing cells in rat.
- SAP specifically eliminates the following:
 - Cholinergic neurons of the basal forebrain
 - Medial septum
 - Diagonal band of Broca
 - Nucleus basalis of Meynert
 - Purkinje neurons of the cerebellum

Effective Tool to Study Behavior

Permanent and selective removal of cholinergic forebrain neurons makes SAP an important animal model for the study of the following:

- Behavior (cholinergic forebrain function)
- Neuronal loss (*e.g.* Alzheimer's disease)
- Plasticity of other systems in response to loss of cholinergic neurons
- Replacement therapy
- Drug effects and dependence [18, 19, 56-58].

C. Hypothesis, Prediction and Aims

Hypothesis

Inhibition of GABA_A receptors by the inhibitory effect of DU-14 on the steroid sulfatase enzyme to increase endogenous levels of sulfated DHEA would counteract the effect of cholinergic lesion to the hippocampus and may enhance retention of passive avoidance memory in rats

Prediction

Chronic administration of DU-14 can enhance retention of passive avoidance memory in rats after selective lesioning of cholinergic neurons of the medial septum

Aim 1

This tested the effect of footshock intensity on memory retention in rats. After selective lesion of cholinergic neurons of the medial septum, rats were randomly assigned to three footshock groups and tested for memory retention. We predicted that an increase in footshock intensity would result in an increase in memory retention in CSF but not SAP lesioned rats. This would signify that SAP lesioning would impair footshock memory retention in rats.

Aim 2

We tested the effect of chronic DU-14 administration on retention of footshock memory function in rats with selective lesion of cholinergic neurons of the medial septum. Rats from footshock levels were administered either DU-14 or Vehicle controls and tested at

the retention phase. We hypothesized that DU-14 would enhance memory for footshock intensity in both CSF and SAP rats. Results obtained displayed DU-14's functional antagonistic property at the GABA receptor, potentiating memory function in SAP animals.

II. EXPERIMENTALS

A. Materials and Equipments

1. Facilities

Laboratories

- Mellon Hall of Science, Duquesne University: Rooms 416A & 454
- Bayer School of Natural and Environmental Sciences, Duquesne University: Animal Facility
- Salk Hall, University of Pittsburgh, Rooms 1005, 1006, 1015 and 1016

Office

- Mellon Hall of Science, Duquesne University: Room 417

2. Animals

Sprague-Dawley male rats

Hilltop lab Animal Inc. Scottsdale, PA

3. Chemicals and drugs

[H³] acetyl-CoA

Sigma Inc., St. Louis, MO

192 IgG Saporin (SAP)

Chemicon, Temecula, CA

Acetonitrile

Sigma Inc., St. Louis, MO

Acetyl-CoA

Sigma Inc., St. Louis, MO

Choline chloride (C₅H₁₄ONCl)

Sigma Inc., St. Louis, MO

Corn Oil

100% pure Mazola

Shop and Save, Pittsburgh PA

Disodium phosphate (Na₂HPO₄)

Sigma Inc., St. Louis, MO

EcoLume™ Liquid scintillation fluid
MP Biomedicals, Scottsdale, OH

Econofluor scintillation cocktail
PerkinElmer Life And Analytical Sciences, Inc., IL

EDTA (Ethylene diamine Tetraacetic acid)
Fisher Chemicals

Halothane, USP / Isoflurane, USP
Abbott Laboratories, North Chicago, IL

Ibuprofen Sodium
Sigma Chemical Co., St Louis, MO

Pentobarbital Sodium
Sigma Chemical Co., St Louis, MO

Physostigmine (eserine salicylate)
Sigma Inc., St. Louis, MO

Sodium chloride (NaCl)
Sigma Inc., St. Louis, MO

Sodium phosphate monobasic monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$)
Sigma Inc., St. Louis, MO

Sodium tetraphenylborone
TCI America, Portland, OR

Triton® X-100
Sigma Inc., St. Louis, MO

4. Materials

BD disposable syringes (1.0cc)
Becton Dickson and Company, Franklin Lakes, NJ

DC Protein Assay kit
Bio-Rad laboratories, Hercules, CA

Disposable Scintillation Vials
Fisher Scientific, Pittsburgh, PA

MLA Precision pipette tips (10, 20-250 and 20-1000 μ l)
Fisher Scientific Pittsburgh, PA
Monosof Black Nylon Suture
4-0. 3/8: 19mm
Tyco Health Care, Norwalk, CT

Precision glide[®] disposable syringes needles (21, 23 and 26 gauge)
Becton Dickson and Company, Franklin Lakes, NJ

Pipettors, Gilson Pipetman (P0.5, P10, P20, P200, P1000 and P5000)
Fisher Scientific Pittsburgh, PA

5. Equipments

1209 Rackbeta liquid scintillation counter
LKB Wallac

Brinkmann Bottle top Dispenser
Brinkmann instruments, Inc.

DECpc 433sx LP computer (integrated with Gemini System)
Digital Equipment Corporation, Maynard, MA

Gemini Passive Avoidance System
San Diego Instruments, Inc., San Diego, CA

Stereotaxic frame
Stoelting, Wood Dale, IL

VirSonic 475 ultrasonic tissue disruptor
VirTis

6. Computer Software

Gemini Avoidance Software
San Diego Instruments, San Diego, CA

Graph pad prism version 3.02
Graph pad software, San Diego, CA

Graph pad prism version 5.01
Graph pad software, San Diego, CA

Microsoft word& Excel 2003
Microsoft Corporation, Orem, UT

B. Methodology and Procedures

1. Animal Condition

All procedures involving the use of animals were in accord with standards established by the Animal Welfare Act and approved by the Institutional Animal Care and Use Committee of Duquesne University. Male Sprague-Dawley rats weighing approximately 275 grams were purchased from hilltop lab Animal Inc. (Scottsdale, PA) and individually housed in a well ventilated, temperature and humidity controlled facility (22-25 degrees centigrade, 50-75% humidity). A standard 12 hour light and 12 hour dark cycle was maintained. Standard laboratory rat chow and water were available *ad libitum*. The animals were allowed a minimum period of five days to acclimate to the housing conditions, before any experiments were performed.

2. Animal Surgery

Male Sprague-Dawley rats weighing approximately 300 grams were anesthetized with pentobarbital (50mg/kg: i.p., of a 50mg/ml stock solution), shaved and then placed in a stereotaxic frame (Stoelting, Wood Dale, IL). An incision was made exposing the dorsal aspect of the skull and a small hole, 2mm in diameter was drilled, through which a stainless steel cannula was placed into the medial septum, using coordinates from Bregma: AP+0.2mm L 0.0, DV-5.4mm. Animals received either SAP, 0.22 μ g/ μ l or CSF (sham control) 1 μ l infused into the medial septum over 5 min at a rate of 0.2 μ l/min. Following infusion, the cannula was left in place for 5 min to allow diffusion into the tissues before the withdrawal of the cannula. The incision was closed and rats were

administered Ibuprofen (1 ml/kg i.p., of a 10mg/ml stock solution). Rats remained anesthetized throughout the surgery and were allowed to recover for at least one week.

3. Passive Avoidance Testing

To assess memory retention, a Gemini Avoidance System (San Diego instruments) was used with a modified passive avoidance paradigm. The avoidance apparatus consisted of a box (53x53x32cm) with 2 compartments connected by an opening with a computer controlled sliding door. The compartment in which the rats were placed was illuminated, while the other compartment remained dark throughout the experiment.

There were three stages of the behavioral testing: the acclimation phase, the acquisition phase and the retention phase. During the acclimation trials that preceded the acquisition phase, rats were allowed to explore the apparatus. The latency period, which was the time it took for the rat to cross into the dark compartment, was recorded. During the acquisition trials, rats were placed in the lighted compartment. After five seconds of adaptation, the rats were given a maximum trial duration of 300 sec to cross to the darkened chamber. If the animal entered the dark compartment, the sliding door closed and a mild footshock of either 0 mA, 1.0 mA or 1.25 mA for 1 sec was delivered. The 1.0 mA footshock was selected based on previous studies in our laboratory, 1.25 mA was a mild increase while the 0 mA was used as a negative control. The rat was then removed from the chamber and tested again after 5 min. This procedure was repeated until the animal spent 300 sec in the lighted chamber two consecutive times within a maximum of 5 trials. The number of trials to reach criterion was recorded. After the acquisition trial,

starting from the second day, the animal was administered either DU-14 (30mg/kg, i.p.) or corn oil (1ml/kg, i.p.) for 6 consecutive days. 3 hours after drug administration on the last day of dosing, the animal was again placed in the lighted compartment and the crossover latency recorded. If a rat did not enter the dark compartment within 600 sec, it was removed from the apparatus, and the latency recorded as 600 sec. Memory retention was assessed as an increased crossover latency period during the second trial.

4. ChAT (Choline Acetyltransferase) Assay

At the completion of behavioral training, all animals were processed for the quantification of ChAT activity in the frontal cortex and hippocampus. Animals were anesthetized with 5% halothane or isoflurane in oxygen, decapitated, brains removed, and tissues from the frontal cortex (FX), medial septum (MS), and hippocampus (HIP) dissected, frozen at 80°C and processed at a later time. Tissues were not pooled. On the day of the assay, frozen tissues were thawed at 4°C and dissociated by sonication in a medium containing EDTA (10mM) and Triton X-100 (0.5%) and diluted to a concentration of 10µl/mg tissue. An aliquot of each sample was used for the determination of total protein [58]. Three 5µl aliquots of each sample were incubated for 30 min in a medium containing 0.25 mM [H^3]-Acetyl-CoA, 50 mM sodium phosphate buffer (pH 7.4); 300 mM sodium chloride, 10 mM choline chloride, 10 mM EDTA and 0.2 mM physostigmine sulfate were used. The reaction was stopped by adding 4ml of 10 mM sodium phosphate buffer (pH 7.4) followed by the addition of 1.6 ml of acetonitrile containing 5mg/ml tetraphenylboron in scintillation vials. The amount of [H^3] – acetylcholine produced was determined by adding 8 ml of EconoFluor scintillation

cocktail and counting total cpm in the organic phase using an LKB beta-counter. Background was determined using identical tubes to which no sample was added. For each sample, the three reaction tubes containing sample were averaged and the difference between total cpm and background cpm was used to estimate the total amount of ACh produced per sample. The ChAT enzyme activity was expressed as pmol acetylcholine production/h/ μ g protein. All results obtained from rats associated with values either below 62.5% mean normal ChAT activity in the frontal cortex (CSF/FX), or above 62.5% mean normal ChAT activity in the hippocampus (CSF/HIPP) were excluded from the entire study.

5. Statistical Analysis

All analyses were performed using the GraphPad Prism 3.02 version while all graphs were drawn using GraphPad Prism 5.01 version. Differences in ChAT activity in the HIPP and FX of SAP treated and control animals were compared using the one-way ANOVA with a Neuman-Keuls post-hoc test. Differences in crossover latency were determined by statistical analysis utilizing the Student's t-test, Kruskal-Wallis analysis with a Dunnett's test post-hoc or a two-way ANOVA with surgery and footshock intensity as parameters. Significant differences were interpreted as $p < 0.05$. The effect of frontal cortex lesioning on acclimation crossover latency was examined using the correlation analysis.

6. Preparation and Administration of Drugs

DU-14 was prepared by suspending 30mg of crystals in 1 ml of corn oil. Ibuprofen solutions were prepared by dissolving 1g in 100mls of deionized water. The route of administration for all drugs was intraperitoneal (IP) using a 23 or 21 gauge needle for the administration of DU-14 and a 26 gauge needle for the administration of corn oil, pentobarbital and Ibuprofen. 5% halothane or isoflurane in oxygen was administered via inhalation.

7. Animal Studies

- a. Effect of footshock on memory retention
- b. Effect of DU-14 on memory retention
- c. Effect of DU-14 on locomotor activity

III. RESULTS AND DISCUSSION

A. Results

1. The effect of 192 IgG-Saporin (SAP) infusion on choline acetyltransferase enzyme (ChAT) activity in frontal cortex and hippocampus

After behavioral studies, rats were anesthetized and decapitated. One frontal cortex and two hippocampal tissues were collected from each rat brain. The tissues were immediately frozen and later analyzed for ChAT activity. ChAT activity was measured to determine presence or absence of cholinergic neurons in the frontal cortex and hippocampal tissues after SAP infusion. A significant decrease in enzyme activity was observed in hippocampal tissues from rats infused with SAP compared to control animals ($p < 0.0001$; Figure 8). There was no significant change in ChAT activity in frontal cortex tissues in SAP treated animals ($p > 0.05$). All results obtained from rats associated with values either below 62.5% mean normal ChAT activity in the frontal cortex (CSF/FX), or above 62.5% mean normal ChAT activity in the hippocampus (CSF/HIPP) were excluded from the entire study.

Figure 8: ChAT activity (Corn oil treated rats)

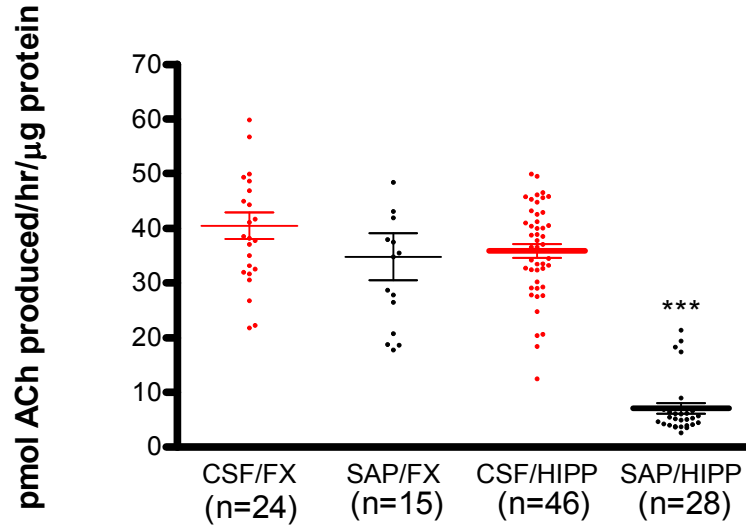


Figure 8: ChAT activity (Corn oil treated rats)

Scatter plots show the effect of SAP lesions on ChAT activity in the frontal cortex (SAP/FX) and hippocampus (SAP/HIPP) relative to control (CSF/FX and CSF/SAP). Infusion of 192 IgG-Saporin produced a significant decrease in ChAT activity in neurons projecting to the hippocampus but not the frontal cortex. Lines show group mean \pm SEM. Individual data points are also shown. All data were analyzed by one-way ANOVA ($F= 63.15$; 3, 109 df; $p<0.0001$) followed by a Newman-Keuls Multiple Comparison test where significance was defined as $p<0.001$, and *** represents a significant decrease in the SAP/HIPP group from all other treatment groups. There was an 80.3% decrease in ChAT activity in hippocampus of SAP versus CSF rats (7.07 ± 0.98 versus 35.90 ± 1.26 pmol ACh produced/hr/ μ g protein; $p<0.001$).

2. The effect of SAP lesioning on acclimation/ locomotion

To assess whether SAP lesioning had an effect on exploratory behavior or locomotor activity, seven days following intraseptal infusion of either SAP or CSF control, rats acclimated to a standard passive avoidance apparatus. There was a significant increase in the acclimation crossover latency in SAP treated animals (Figure 9).

Figure 9: Effect of SAP lesioning on acclimation

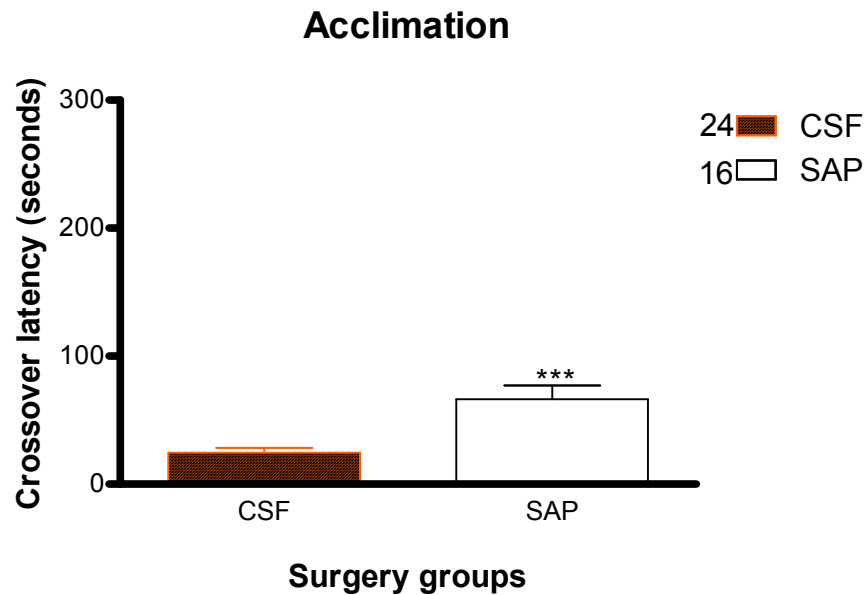


Figure 9: Effect of SAP lesioning on acclimation

Graph shows the crossover latency in seconds versus the treatment groups. A significant increase in crossover latency was seen for SAP treated animals. Bars represent the mean \pm SEM. All data were analyzed using the Student's t-test ($p < 0.0001$).

3. Effect of shock intensity on memory acquisition

In this set of experiments, animals acquired memory for footshock of different intensities. This test was also carried out to determine the optimal shock intensity for the study and the effect of shock intensity on the acquisition and retention phases of the passive avoidance test. Following acclimation, rats were randomly assigned to three different groups (0 mA (no shock), 1.0 mA, and 1.25 mA). The criterion for selection is stated in the methods section. Figure 10 shows the number of trials to reach criterion for control and SAP rats from the 1.0 mA and 1.25 mA shock levels. Since there was no memory to acquire at 0 mA, no representative graphs were shown. The results show no significant difference in acquisition of memory between SAP and control rats for footshock at either 1.0 mA or 1.25 mA shock intensities (Figure 10). However, no animal in the SAP/1.25 mA group required more than a single shock to reach criterion.

Figure 10: Effect of shock intensity on memory acquisition

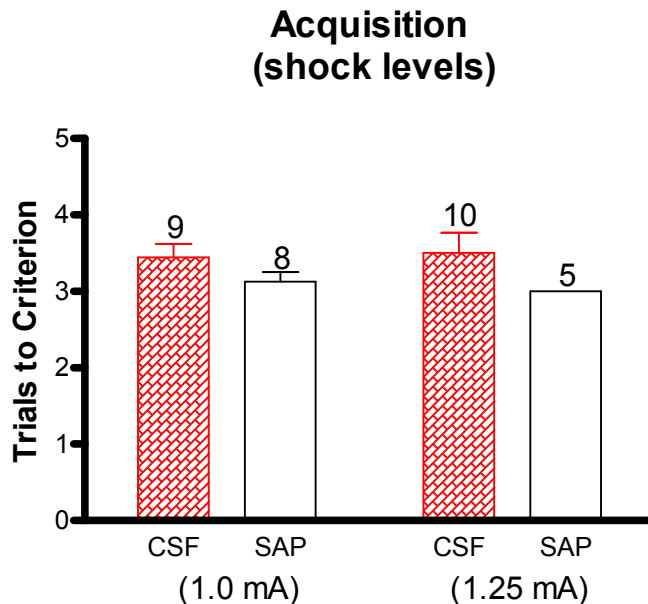


Figure 10: Effect of shock intensity on memory acquisition

The number of trials to reach criterion was compared for two different shock intensities: 1.0 mA and 1.25 mA. Bars represent the mean \pm SEM. A two-way ANOVA found neither a SAP nor a shock intensity effect on acquisition of footshock memory (Surgery $p = 0.066$; Shock intensity $p < 0.87$; interaction $p = 0.68$).

4. Effect of footshock on retention

CSF and SAP rats from the acquisition phase were dosed for six days with corn oil, and then tested on the sixth day of dosing for retention of footshock memory. Rats assigned to the 0 mA footshock group also learned the maze without exposure to footshock. They were passed through the apparatus in a similar fashion as the other groups allowing for five minute inter-trial intervals for a maximum of 3 trials. During the retention test, all rats in the 0mA footshock intensity group crossed over in less than 100 sec similar to results observed during the acclimation phase. There was a non-significant increase in retention crossover latency in SAP rats at all shock levels, 0 mA, 1.0 mA and 1.25 mA with p values of 0.07, 0.1672 and .00992 respectively. An increase in shock intensity produced an increase in retention crossover latency in both CSF and SAP rats. In both groups however, there were no significant differences in crossover latency between rats administered 1.0mA and 1.25mA footshock (Figure 11).

Figure 11:

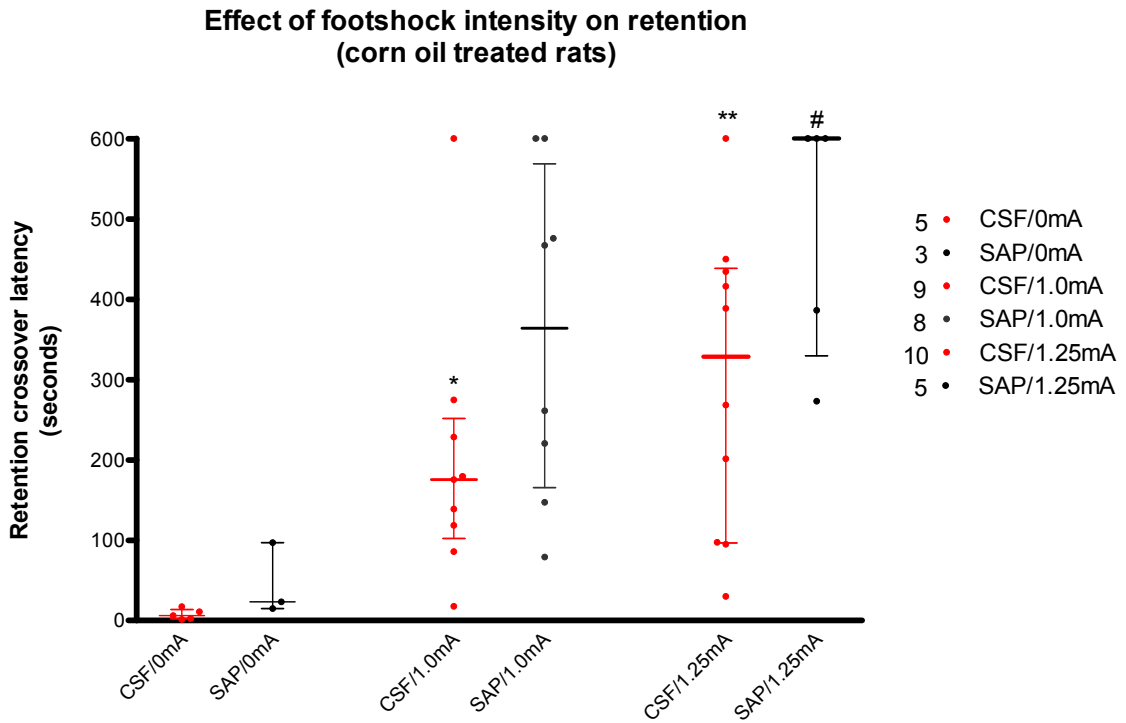


Figure 11: *Effect of footshock intensity on retention*

Scatter dot plots show the effect of footshock intensity on memory retention. Lines show the median with interquartile range. Individual data points are also shown. All data were analyzed by a Kruskal-Wallis non-parametric test ($p < 0.0005$) followed by a Dunn's multiple comparison test where * and ** represent significant differences from CSF/0mA; $p < 0.05$ and $p < 0.05$ respectively and # represents a significant difference from SAP/0mA; $p < 0.01$.

5. The effect of 192 IgG-Saporin (SAP) infusion on choline acetyltransferase enzyme (ChAT) activity on rats' frontal cortex and hippocampal tissues (DU-14 rats)

ChAT activity was measured in DU-14 treated rats to assess the degree of cholinergic innervation of the frontal cortex and hippocampus following SAP infusion. One frontal cortex and two hippocampal tissues were collected from each rat brain. SAP infusion into the medial septum produced a significant decrease in enzyme activity in the brain hippocampal tissues ($p < 0.0001$; Figure 12). There was also a significant effect of SAP lesion on frontal cortex ChAT activity when compared to CSF frontal cortex group ($p < 0.05$). All results obtained from rats associated with values either below 62.5% mean normal ChAT activity in the frontal cortex (CSF/FX), or above 62.5% mean normal ChAT activity in the hippocampus (CSF/HIPP) were excluded from the entire study.

Figure 12: Effect of 192 SAP infusions on ChAT activity in frontal cortex and hippocampal tissues (DU-14 rats)

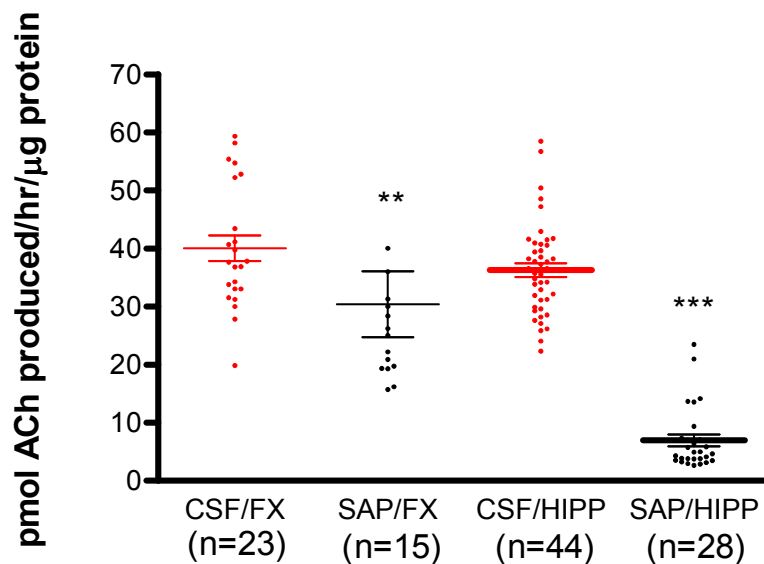


Figure 12: Effect of 192 IgG-Saporin (SAP) infusions on choline acetyltransferase enzyme (ChAT) on rats' frontal cortex and hippocampal tissues (DU-14 rats)

Scatter dot plots show the effect of SAP lesions on ChAT activity in the frontal cortex (SAP/FX) and hippocampus (SAP/HIPP) relative to control (CSF/FX and CSF/SAP). Infusion of 192 IgG-Saporin produced a significant decrease in ChAT activity in neurons projecting to the hippocampus but not the frontal cortex. Lines show group mean \pm SEM. Individual data points are also shown. All data were analyzed by one-way ANOVA ($F=52.02$; 3, 106 df; $p<0.0001$) followed by a Newman-Keuls Multiple Comparison test where significance was defined as $p<0.001$, and *** represents a significant decrease in the SAP/HIPP group from all other treatment groups. There was an 80.9% decrease in ChAT activity in hippocampi of SAP versus CSF rats (6.95 ± 1.02 versus 36.31 ± 1.20 pmol ACh produced/hr/ μ g protein; $p< 0.001$). There was also a 24.1% decrease in ChAT activity in the frontal cortex of SAP rats when compared to similar tissues in CSF rats (30.07 ± 5.70 versus 40.41 ± 2.22 pmol ACh produced/hr/ μ g protein; ** $p< 0.001$).

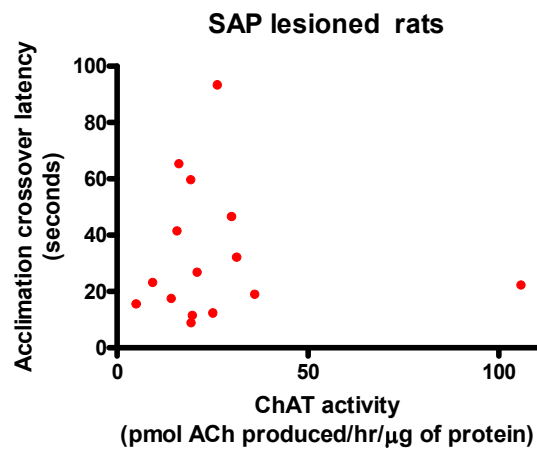
6. Effect of frontal cortex lesion on acclimation/ locomotion in rats

A significant decrease was seen in the frontal cortex of SAP treated animals when compared to those of CSF infused rats. Thus, there was a possibility for the loss of cholinergic tone in the frontal cortex that could affect rat's locomotor activity. A spearman's correlation analysis was performed to predict the relationship between cholinergic denervations of specific regions of the brain and behavioral performance. All SAP rats were first examined and then further grouped based on lesioned region. Groups

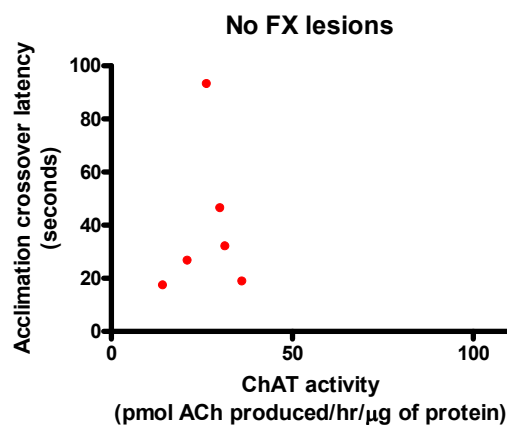
were SAP infused rats with lesions in the hippocampus but not the frontal cortex (No FX lesion) and SAP infused rats with lesions in both the hippocampus and frontal cortex (FX+HC lesion). SAP lesioning in the frontal cortex or hippocampus was defined as 62.5% below mean normal ChAT activity of CSF/FX or CSF/SAP groups respectively (Figure 12). Extent of lesioning showed no correlation to acclimation crossover latency (sr=0.1036, 0.2000 and -0.4524; Figure 13).

Figure 13: Effect of Frontal cortex lesion on acclimation/locomotion

A



B



C

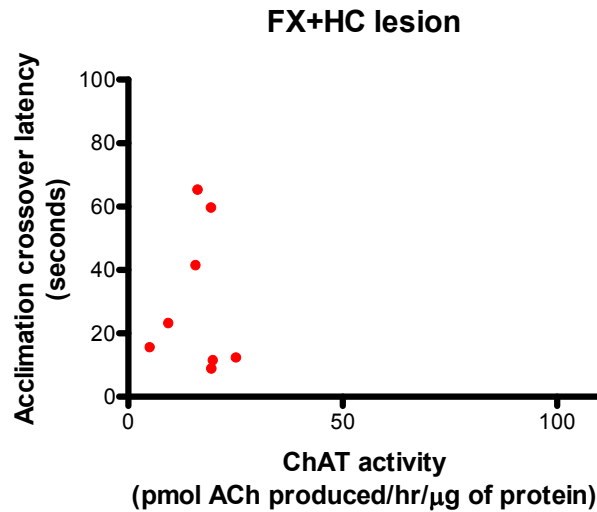


Figure 13: *Effect of frontal cortex lesion on acclimation/ locomotion in rats*

Scatter plots show relationship between the ChAT activity and acclimation crossover latency. All data were analyzed by the Spearman's Rho non-parametric correlation analysis. A: In all SAP treated rats, there was an insignificant correlation seen; ($sr=0.1036$, $p=0.7134$). With rats further divided based lesioned region of the brains, B: In rats No FX lesion rats an insignificant correlation was observed ($sr=0.2000$, $p=0.7139$). Likewise in rats with lesions in both the frontal cortex and the hippocampus, C: an insignificant correlation was observed ($sr=-0.4524$, $p=0.2675$).

7. Effect of DU-14 on memory retention after 1.0mA footshock administration

To determine the effect of DU-14 on retention memory, rats were tested using passive avoidance as described previously. Rats were tested for memory retention after 6 days of DU-14 dosing. As seen in Figure 14, treatment with DU-14 significantly increased crossover latency in SAP treated animals ($p=0.0476$). There was also a non-

significant increase in the crossover latency in control animals treated with DU-14 ($p=0.1563$).

Figure 14:

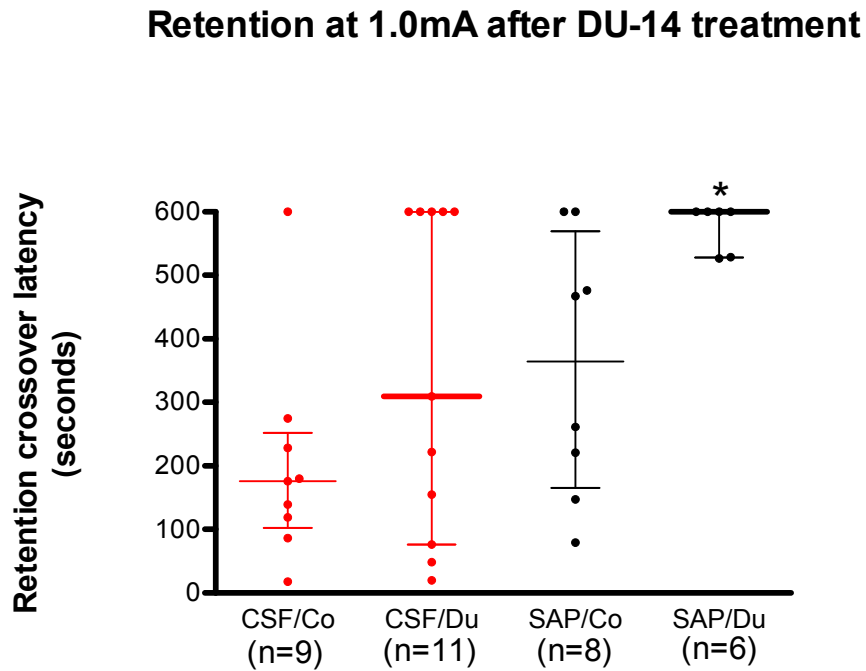


Figure 14: *Effect of DU-14 on memory retention after administration of 1.0mA footshock*

Scatter dot plot shows the effect of DU-14 administration on memory. Lines show the median with interquartile range. Individual data points were also shown. All data were analyzed by a Kruskal-Wallis non-parametric test ($p=0.0476$) followed by a Dunn's multiple comparison test where * represents a significant difference from SAP/Co; $p<0.05$.

8. Effect of DU-14 on memory retention after 1.25mA footshock administration

The effect of DU-14 on memory retention was also tested in rats after 1.25mA footshock administration. Rats were tested for memory retention after six days of DU-14 dosing. DU-14 produced no significant change in the crossover latency in either control or SAP animals.

Figure 15: Effect of DU-14 on memory retention after administration of 1.25mA footshock

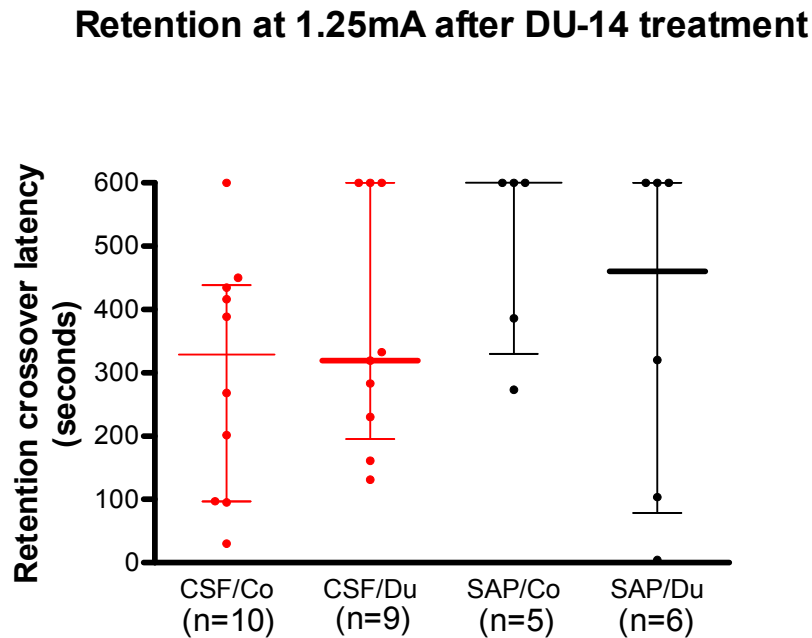


Figure 15: Effect of DU-14 on memory retention after administration of 1.25mA footshock

Scatter dot plot shows the effect of DU-14 administration on memory. Lines show the median with interquartile range. Individual data points were also shown. All data were analyzed by a Kruskal-Wallis non-parametric test ($p=0.4029$). No significances were seen among groups.

9. Effect of DU-14 on locomotion

One possible explanation for the DU-14 effect seen in Figure 14 was a possible effect of the drug on locomotor activity. Perhaps after 6 days of dosing, rather than enhance memory retention, DU-14 treatment affected locomotor activity. It was therefore necessary to perform the acclimation test with a separate group of rats after dosing with DU-14. Rats (CSF and SAP) dosed with DU-14 for 6 days one week after surgery showed no significant differences from untreated rats (CSF and SAP) in crossover latency when tested during the acclimation phase, before acquisition of footshock. No DU-14 effect was seen on spontaneous locomotor activity (Figure 16a). Another set of experiment was carried out to determine the effect of DU-14 on locomotion after the retention phase. Rats administered no footshock during the acquisition phase were tested for 0 mA footshock memory retention after six day of dosing with either DU-14 or corn oil (vehicle). There was no significant difference between rats treated with DU-14 and corn oil (Figure 16b).

Figure 16a: Effect of DU-14 on locomotion (acclimation phase)

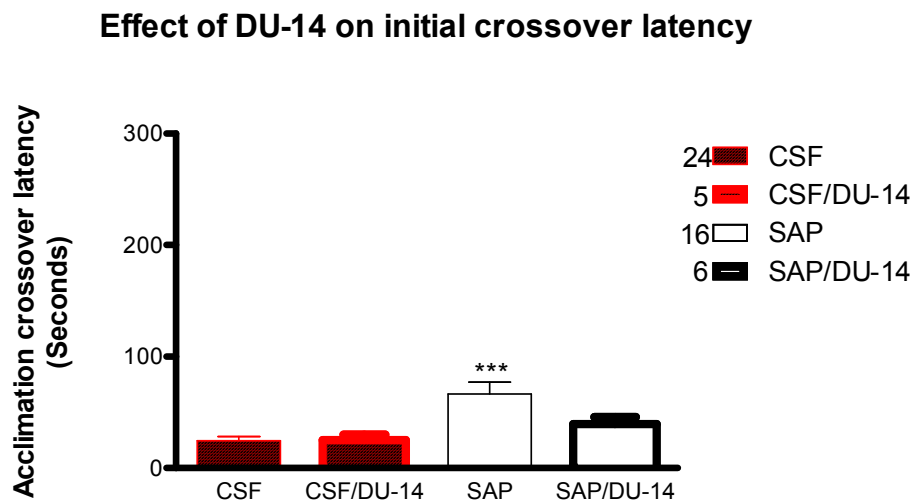


Figure 16a: Effect of DU-14 on locomotion (acclimation phase)

Each bar represent the mean \pm SEM. There was no significant DU-14 effect on spontaneous locomotor activity. All data were analyzed Kruskal-Wallis test ($p = 0.0003$) followed by a Dunn's multiple comparison test where *** represents a significant difference from CSF; $p < 0.0001$. The significant difference seen between CSF and SAP groups has previously been established in Figure 9.

Figure 16b: Effect of DU-14 on locomotion (retention phase)

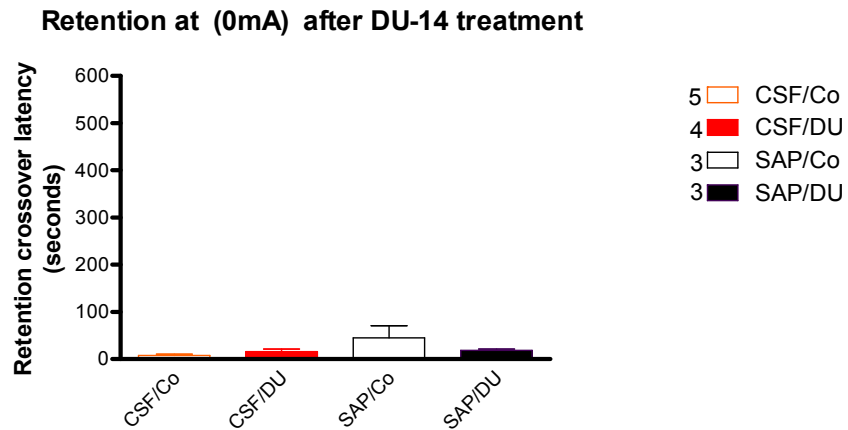


Figure 16b: Effect of DU-14 on locomotion (retention phase)

Each bar represent the mean \pm SEM. There was no significant difference in crossover latency between rats treated with DU-14 and corn oil. All data were analyzed Kruskal-Wallis test ($p=0.1468$).

B. Discussion

In this study, we showed that infusion of SAP decreased cholinergic innervation of the hippocampus, a model that has been used in the past to mimic memory impairment as seen in Alzheimer's disease [54, 56]. We also showed that SAP lesioning affected spontaneous locomotor/exploratory behavior, an effect that has not been previously reported. Nevertheless, the change in locomotor activity did not totally impair exploratory behavior because rats crossed over to the darkened chamber in less than 100 seconds during the acclimation phase of the experiments.

In the past, various footshock intensities ranging from 0.2 to 1.0 mA had been used in passive avoidance experiments [5, 47-49, 59, 60]. Therefore, it was necessary to determine the effect of footshock intensity on memory acquisition and retention, and how increased shock intensity would affect retention crossover latency. There was a non-significant decrease in the number of footshocks required by SAP rats to acquire the task in both the 1.0mA and 1.25mA groups compared to CSF rats. It was, however, interesting that, without exception, all SAP, but not all control 1.25mA rats required no more than a single footshock to reach criterion. The effect observed with the SAP rats requiring fewer footshocks to reach criterion, though statistically insignificant, nonetheless suggests the possibility of either heightened sensitivity to the footshock or enhanced aversion if the perceived intensity was the same as for control animals. In addition, since SAP treatment significantly increased crossover latency, both responses to SAP treatment might have a synergistic effect to increase crossover latency at the higher shock intensity.

To determine the effect of footshock on memory retention, following the acquisition phase, rats were tested for retention of footshock memory on the sixth (6th) day of dosing with corn oil. Rats administered 0 mA footshock exhibited no significant difference in the retention (figure 11) and the crossover latency remained similar to latencies observed during acclimation phase. This suggests that any increase in retention crossover latency after shock (1.0/ 1.25 mA) administration was as a result of memory for actual shock.

Rats in the 1.0 mA footshock group had over a five fold increase in retention crossover latency when compared to the acclimation phase in both CSF and SAP lesioned rats. There was also an increase in retention crossover latency in the SAP/1.0mA group when compared to the CSF/1.0mA. This increase was however, not significant. Surprisingly, the results of these experiments were inconsistent with previous findings. Samreen Arshad in her study reported that intraseptal infusion of SAP into the medial septum produced a significant reduction in retention crossover latency in rats [61]. Several factors, however, may have contributed to the differing results. First, the rats in Ms. Arshad's study did not receive daily injections. Intra-peritoneal injection is an aversive stimulus and thus may have attenuated the expected impairment in retention in the passive avoidance experiment [58]. Stress is known to cause elevations in neuroactive steroid levels, in particular a derivative of corticosterone, the neurosteroid tetrahydrodeoxycorticosterone (THDOC), as well as alloprenanolone [62]. Belz et al. [63] demonstrated that daily stimulation with an IP injection of saline would act as a mild stressor and induce elevations of plasma corticosterone. Moreover, Fitz et al. [58] reported that IP injection prior to training eliminated the impairment induced by

hippocampal cholinergic lesion on acquisition of a DMP task. These authors concluded that the introduction of a mild stressor reduced impairment produced by the hippocampal cholinergic lesion. These results were consistent with the relative lack of effect of hippocampal SAP lesion on acquisition of the more stressful MWM task [64]. The study demonstrated that an aversive stimulus consisting of daily injection would mask expected memory impairment caused by SAP infusion [58]. Similarly, for rats administered a higher footshock intensity of 1.25 mA, there was over a 10 fold increase in retention crossover latency (Figure 11) when compared to acclimation crossover latency (Figure 9). The increase seen in SAP/1.25mA rats when compared to the CSF/1.25mA rats was not significant, which again may have been due to the daily injection of rats over six days.

Considering the capacity of the brain to adjust for alterations in neuronal activity, it is possible that with a chronic loss of cholinergic tone in the hippocampus following SAP infusion, neuronal remodeling may compensate to support GABAergic and/or glutamatergic neurotransmission to levels that preserve memory function at normal or near normal levels [65]. Thus an alternative explanation for the SAP effect and/or trend observed in these experiments may be due to the possibility that damage to one memory system might enhance learning mediated by another [66] as demonstrated by Matthew and Best where they showed that fimbria/fornix lesion facilitated learning in a non-spatial task compared to non-lesioned rats. Such that the intact animal is biased to process space because of functioning spatial cognitive map whereas the lesioned animal suffers no such bias, relying on the intact region in the brain. This explanation supports the view that multiple memory systems compete and are integrated to form a cognitive

response [67], and as an example, regions of the brain such as the amygdala play a role in fear memory retention in the passive avoidance paradigm.

It may be worthwhile in the future, in order to eliminate the aversive aspect of IP drug administration, to design a study that would use a less stressful route to administer the drugs such as a single depot injection.

The increase in footshock intensity from 0 mA to 1.0 mA produced a significant increase in retention crossover latency. There was a further increase in latency with administration of 1.25 mA footshock. However, the increase in retention crossover latency from 1.0 mA to 1.25 mA was not significant. A similar trend was seen with SAP treated rats with increased footshock intensity resulting in a corresponding increase in crossover latency. Since there was no significant impairment of ChAT activity in the frontal cortex in corn oil treated rats, our data support the role of the hippocampus in spatial learning and working memory as opposed to fear learning in our study [68, 69].

The next set of experiments was carried out in order to determine the effect of DU-14 on footshock memory retention. An assay for the ChAT enzyme was performed on the hippocampi and frontal cortex from rats in this group to determine whether cholinergic lesion was successful in those specific regions of the brain. Results demonstrated appropriate lesion in the hippocampus, however, there was a significant 24.1% reduction in ChAT activity in the frontal cortex as well. This finding suggests the possibility that retention latency may have been affected as a result of impairment of frontal cortical function in addition to the hippocampus. After examining the correlation between the frontal cortex lesion and acclimation crossover latency (Figure 13), the

decrease in mean ChAT activity in the frontal cortex tissues of SAP treated rats did not seem to affect rats' exploratory behavior (Figure 13).

After 1.0mA footshock, SAP treated rats administered DU-14 had a significant increase in crossover latency when compared to control rats. Previous articles have demonstrated that animals chronically treated with DU-14 had increased plasma DHEAS and enhanced hippocampal acetylcholine release. There was also a reduction of scopolamine induced amnesia utilizing a passive avoidance paradigm [70, 71]. The steroid sulfatase inhibitor effect had previously been demonstrated in rats with scopolamine induced amnesia but not untreated animals [70, 72]. By inhibiting the metabolism of DHEAS to DHEA, DU-14 could increase endogenous levels of DHEAS which would then decrease GABAergic inhibitory tone on cholinergic neurons of the basal forebrain [5]. This could explain the increase in hippocampal ACh and the observed decrease in scopolamine induced amnesia observed in previous studies [70]. Similarly, Arshad in her study showed that DHEAS at 10mg/kg would diminish memory deficits in rats after selective lesion to the hippocampus but had no effect in control rats [61]. This result further suggests and supports the finding that DU-14 has the potential to enhance memory function in patients with cholinergic deficits e.g. in Alzheimer's disease. A possible advantage of DU-14 administration over DHEAS is that it not only elevates the endogenous DHEAS levels but other steroids such as estrone sulfate, pregnenolone sulfate and cholesterol sulfate which have also been implicated in memory enhancement. DU-14 may be an effective therapeutic tool when co-administered with other memory enhancing medications which differ in their mechanism of action. This would lower individual medication dose as well as minimize observable side effects.

At the 1.25 mA footshock level, DU-14 had no significant effect on retention crossover latency in either control or SAP animals. This result suggests that the level of shock intensity in addition to daily injections may induce a degree of arousal above a level to produce a differential effect in control and SAP treated animals. Interestingly, a similar suggestion was proposed by Carmen Sandi stating that interference with long-term memory formation by the blockade of brain glucocorticoid receptors (GRs) might be effective only for learning situations that involve a moderate stress component, but ineffective when dealing with more traumatic experiences [60]. Consistent with this finding, there are a number of studies in the literature that report different intensities of stressful stimuli can induce either impairment or facilitation of memory [60, 73, 74]. Activation of the physiological stress response results in an elevation of glucocorticoid levels. Given the lipophilic nature of glucocorticoids, they can easily cross the blood-brain barrier and enter the brain where they can influence brain function and behavior via binding to different receptor types [60]. Thus, consistent with the results of the Fitz study, the 1.25mA shock in addition to the daily IP injections could act synergistically to attenuate the SAP effect expected.

Finally, this study demonstrated that DU-14 delayed crossover latency via enhancement of retention, since DU-14 administration was found to neither affect acclimation nor retention crossover latency (Figures 16a & 16b) when footshock was not administered during the experiment.

IV. CONCLUSIONS

The results of these experiments support previous findings that chronic DU-14 administration enhances memory function in rats with cholinergic impairment of the CNS. The memory enhancing properties of DU-14 may potentially enhance memory function in patients with dementia e.g. Alzheimer's disease. DU-14 has the potential to become a therapeutic tool when co-administered with other memory enhancing medication with a different mechanism of action. This would not only lower individual medication dose but minimize observable side effect.

A finding from this study was that SAP had a small but significant effect on locomotor activity. While SAP did not decrease the rats' exploratory behavior, it was shown that SAP treatment decreased spontaneous locomotor activity. Thus, in addition to memory function, the hippocampus may have an effect on motivation and/or spontaneous exploration in rats. The effect of SAP could possibly also be attributed to sensitization of sensory receptors in the footpads.

Finally, aversive stimuli either in the form of daily injection in rats, or by administration of specific footshock intensity (in this case, 1.25mA footshock intensity) was found to attenuate memory differences between SAP and control animals. While these results support previous findings in our laboratory, it would be worthwhile in future research to study SAP treated rats using a passive avoidance paradigm without the aversion produced by daily injections either by administering a single depot injection of DU-14, or a less aversive method of administration such as oral administration.

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