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D-CYCLOSERINE BUT NOT D-SERINE REVERSES MEMORY IMPAIRMENTS IN A SCOPOLAMINE INDUCED MODEL OF ALZHEIMER'S DISEASE

By Charlotte E.K. Eyring

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Towson University
Towson, Maryland 21252

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This is to certify that the thesis prepared by Charlotte Eyring entitled D-Cycloserine but not D-Serine Reverse Memory Impairments in a Scopolamine Induced Model of Alzheimer's Disease has been approved by the thesis committee as satisfactory completing the thesis requirements for the degree Master of Art.

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ABSTRACT

D-CYCLOSERINE BUT NOT D-SERINE REVERSES MEMORY IMPAIRMENTS IN A SCOPOLAMINE INDUCED MODEL OF ALZHEIMER'S DISEASE

Charlotte Eyring

Acting as agonists on the glycine site of the NMDAR system, D-Serine (DS) and D-Cycloserine (DCS) have been described as mediators of hippocampal function and therefore of learning and memory. With degradation of the hippocampus a primary factor observed in Alzheimer's Disease (AD), the present study sought to investigate whether DS and DCS could offer some cognitive benefit to patients with AD. This theory was tested using the Scopolamine (SCOP) model of AD in rats. The Competitive Place Task (CPT) water maze was used to investigate the influence of DS and DCS on learning and memory in both SCOP treated and normal rats. The CPT also allowed for exploration into the nature of the striatum and hippocampus as competing memory systems and the degree to which each of these drugs is influencing them. Results indicated that DCS but not DS was able to reverse the working and spatial memory impairment caused by SCOP. DCS was unable improve long term recall in SCOP animals and DS and DCS alone showed no cognitive benefit in normal subjects. The present study found evidence to suggest that DS may primarily be influencing the striatal but not hippocampal, a theory that is in debate.

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ABBREVIATIONS

AD: Alzheimer's Disease

SCOP: Scopolamine

DCS: D-Cycloserine

DS: D-Serine

NMDAR: N-methyl-D-aspartate receptor system

ACh: Acetylcholine

LTP: Long Term Potential

CPT: Competitive Place Task

PCP: Phencyclidine

D-Cycloserine but not D-Serine Reverses Memory Impairments in a Scopolamine Induced Model of Alzheimer's Disease

Since its discovery in 1906 (Berchtold & Cotman, 1998), Alzheimer's disease (AD) has become the most common and devastating form of mental decline in populations of people over the age of 65 years (Francis, Palmer, Snape & Wilcock, 1999). At present, AD is estimated to affect roughly 5 million people in the United States alone (Butterfield & Pocernich, 2003) and its prevalence is only forecast to increase with time. Some predict that by the year 2050, 1 out of every 85 people worldwide will be stricken with AD (Brookmeyer, Johnson, Ziegler-Graham & Arrighi, 2007). Therefore, developing a better understanding of the biology of Alzheimer's has become a critical necessity within the scientific community.

Although AD has been studied to a great extent, scientists are still far from discovering a specific cause or treatment plan for this devastating disorder. In the present paper, the neuroanatomy of AD is briefly discussed in order to give the reader a better understanding of the anatomy and symptomology of the disease. A previously founded hypothesis and model of AD is then presented followed by an introduction to two relatively novel drugs called D-Cycloserine (DCS) and D-Serine (DS). Evidence suggesting that DCS and DS are not only capable of improving cognition in normal subjects but also of reversing cognitive ailments will be the focus of the literature review on these two drugs. Finally, a research proposal is presented which suggests that DCS and DS may prove to be useful options for the treatment of the memory impairments observed in AD.

Alzheimer's Disease

Alzheimer's disease (AD) is classically distinguished by the particularly destructive manifestation which includes an overall cognitive decline due to irreversible neurodegeneration, and an array of resulting symptoms including those related to memory, cognition, behavior and language (Francis et al., 1999). From a pathological standpoint, AD is characterized by the development of neuritic plaques and neurofibrillatory tangles (Butterfield & Pocernich, 2003, Weinstock, 1999) which lead to neuronal death and eventually atrophy due to the presence of neurotoxic factors (Weinstock, 1999). There are a variety of theories regarding the development of the tangles and plaques which have become synonymous with AD. For example, some researchers have suggested correlations between AD development and the type 1 strain of the Herpes simplex virus (Itzhaki & Wozniak, 2008), the presence of apoE gene (Polvikoski, 1995) and an age related breakdown in myelin (Bartzokis, 2011). However, the most well known theories are the amyloid hypothesis, which suggests that AD develops when amyloid beta deposits result in the development of amyloid plaques in the brain (Hardy & Allsop, 1991) and the Tau hypothesis, which postulates that a protein called Tau causes the development of neurofibrillatory tangles which leads to a breakdown in neuronal communication and subsequently cell death (Goedert, Spillantini, & Crowther, 1991; Chun & Johnson, 2007). However, despite a great deal of research being conducted on AD, universal support for one hypothesis in particular has not been found and a fully beneficial treatment option for symptoms associated with AD has not been discovered. One factor standing in the way of finding a treatment is the global influence AD has on the brain. Unlike many disorders which are localized to one specific

region or neurotransmitter system, AD appears to devastate multiple brain regions and a variety of neurotransmitter systems simultaneously, leaving scientists unable to tease apart the cause from the result. Research has shown that anatomically, AD results in the break down and death of neurons and synapses in most areas of the cerebral cortex including the parietal, temporal, and frontal lobes of the brain (Wenk, 2003). Specific regions important for learning, memory and other higher level functioning such as the hippocampus and neocortex are also severely degraded along with many other regions including the amygdala (Francis et al. 1999).

One of the most prevalent theories regarding the cause of AD points to a defective neurotransmitter system. The Cholinergic Hypothesis of Alzheimer's disease is one of the oldest and most studied theories of AD and suggests that symptoms of AD (especially those related to leaning and memory) may be the result of a breakdown in the cholinergic system (Bartus, 2000; Bartus, Dean, Beer and Lippa, 1982; Francis et al, 1999). More specifically, it is hypothesized that the loss of cognitive function observed in AD patients is the result of degeneration in the cholinergic neurons of brain regions responsible for projecting information to cortical areas (Fransis et al., 1999). This breakdown results in a depletion of a critically important neurotransmitter called acetylcholine (ACh), without which normal communication within the brain cannot be achieved. ACh is known to play a role in hippocampal Long Term Potentiation (LTP), a process necessary for learning and memory, and is also involved in neuronal plasticity in the cortex (Mesulam, 2004). Therefore, a lack of ACh in the brain, especially in regions such as the hippocampus and other higher level regions, would result in an inability to learn, develop and recall memories- all of which are symptoms of AD. Physical evidence for this hypothesis

provides verification of significant deficits in the synthesis of acetylcholine in the brains of patients with AD (Bowen, Smith & White, 1976). The result of a decrease in choline uptake and ACh release (Francis et al. 1999),- a variety of brain regions including the Temporal lobe, hippocampus, and entorhinal cortex have been found to be severely depleted of Ach (Pappas, Bayley, Bui, Hansen & Tai, 2000).

Although deficits in the cholinergic system of patients with AD are indisputable, doubts about the cholinergic hypotheses have arisen due in part to the development of treatments designed to enhance or even reverse cholinergic function via ACh agonists or enzymatic regulation. Results of studies looking at the effectiveness of such drugs have described only limited and short term benefits, many of which result in serious side effect. Nevertheless, developing a model of AD has become critically important. Therefore, several models of AD have been developed with ACh in mind.

Based on the Cholinergic Hypothesis of AD, models of the disease have been developed in which the brain systems of animals or humans are depleted of ACh by means of neurotransmitter antagonist or cholinergic ligation. One ACh depletion model that has been developed uses an ACh antagonist called Scopolamine (SCOP) to study AD. Although some researchers argue that SCOP does not mimic the symptoms of AD closely enough to be considered a valid model, the vast number of systems affected by AD, along with the extent of behavioral symptoms observed make a synthetic model of AD difficult to produce. Proponents of the SCOP model of AD point to the fact that SCOP does effectively reduce ACh activity by blocking muscarinic receptors in many areas associated with AD including the cerebral cortex, hippocampus, thalamus and amygdala (Obonsawin et al. 1998) and therefore is able to produce behavioral and

anatomical symptoms in subjects similar to what is observed in AD patients. Furthermore, from a behavioral standpoint, SCOP is capable of producing AD-like cognitive impairments in both humans and animals. For example, while traditionally AD is considered a disease of memory malfunction, SCOP too has been linked to a variety of memory impairments including face-name retrieval tasks in humans (Sperline et al., 2001), visual recognition performance in monkeys (Turchi, Saunders, & Mishkin, 2004) and impaired acquisition and long term memory of spatial information during the Morris Water maze in rats (Herrera-Morales, Mar, Serrano, & Bermudez-Rattoni, 2007; Amico, Spowart-Manning, Anwyl, & Rowan, 2007; Mogensen et al. 2002; Sutherland, Whishaw & Regehr, 1982). Additionally, SCOP has been reported to cause verbal fluency impairments in the elderly (Sunderland et al. 1987, Molchan et al. 1992) a symptom that is also reported in patients with AD.

Although SCOP has been implicated in the inhibition of several ACh-dependent brain regions, it is its effectiveness at suppressing normal hippocampal function by means of cholinergic interception which has encouraged interest by some memory researchers. SCOP has been documented to selectively inhibit the hippocampus while leaving another critical memory system, the striatum, mostly intact, making it ideal for isolating a specific memory region which is thought to be the root of many of the memory impairments observed in AD patients. Scientific proof of this was produced by Sperling et al. (2001), who used fMRI to scan the brains of normal human participants treated with SCOP during a face-recognition task. The results of the scan demonstrated that participants treated with SCOP were significantly impaired at recognizing faces compared to control; furthermore, this trend was highly correlated with a significant decrease in the

hippocampal but not striatal function while under the influence of SCOP. The results of this study are important because they provide physical evidence that memory impairments observed as a result of SCOP treatment are primarily due to hippocampal malfunction as opposed to other memory regions.

Water maze studies using animals have also been conducted in order to provide evidence that SCOP is disrupting normal cognitive function and memory. In one study, Herrera-Morale et al. (2007) used Scopolamine to test hippocampal involvement in long-term memory formation in rats. To do this, rats received stereotaxic injections of Scopolamine or a control vehicle directly into the dorsal hippocampus. Rats were then given a series of trials over the course of 4 consecutive days in which they were to learn the location of a stationary hidden platform within the water maze. Using a single probe test, the rats' memory was tested again one week after succession of part 1 of the water maze trials. Results indicate that SCOP-treated animals performed significantly worse during the acquisition portion of the trial (Days 1-4) as demonstrated by a more gradual learning curve (or overall increased escape latency time to the platform) compared to controls. Furthermore, when long term memory was tested one week later, SCOP-treated animals took significantly longer to reach the old platform location and crossed the hidden platform location significantly fewer times then controls. Overall, the behavior of the drug-treated rats suggests that SCOP significantly impairs both acquisition of new spatial information and long term memory for spatial information.

Similar results were found by Amico et al. (2007) when they tested the effects of Scopolamine on spatial memory in the water maze. Using simple I.P. injections of SCOP, followed by 5 consecutive days of water maze trails and then a probe test 3 hours after

the last trial on day 5 to test memory recall, Amicao et al. (2007) demonstrated that SCOP-treated animals were significantly impaired at acquisition of new memory, recall of memory, and spatial navigation. Furthermore, the results of this study provide evidence that SCOP can be used to study hippocampal mal function by means of simple I.P. injections.

Although SCOP treatment does not produce a universally precise replica of AD, SCOP treated subjects do demonstrate symptoms similar to those observed in AD patients. SCOP acts as a potent ACh antagonist, able to block normal function of many of the same regions that are affected by AD including the hippocampus (Sperling et al., 2001). The result of SCOP treatment is often times related to an inability to acquire new information or recall what has already been learned (Amico et al., 2007; Herrera-Morale et al., 2007), both of which are defining features of AD. Therefore, despite the fact that the SCOP model of AD has not be universally accepted as a 'true' model of Alzheimer's, it remains similar enough from an anatomical and a behavioral standpoint to be studied in greater depth. However, while using a model of AD which is produced by mimicking the ACh deficits in AD seems relevant, the lack of evidence in support of using the cholinergic system as a model of treatment suggests that there may be a superior means of reversing the memory impairments observed in the SCOP model of AD. One system that has been implicated in learning and memory, LTP in the hippocampus, and cortical plasticity is the NMDA receptor system and may therefore be able to combat many of the symptoms related to the cognitive side effects of AD. Additionally, two relatively novel NMDA agonists, D-Cycloserine and D-Serine, may prove to be the ideal for the SCOP model of AD.

The NMDA Receptor System

The N-methyl-D-aspartate receptor system (NMDAR) is arguably the most prevalent excitatory neurotransmitter system in the mammalian central nervous system (Monahan, Handelmann, Hood & Cordi, 1989). On a molecular level, NMDAR is activated though a complex system involving depolarization of its ion linked receptor channel by simultaneous activation of the glutamate and glycine sites on the receptor and removal of the magnesium channel blockade. The result is a rapid influx of calcium ions into the cell causing a series of intercellular changes thereby allowing learning to transpire (Baxter, Lanthorn, Frick, Golski, Wan & Olton, 1994). The NMDA receptor system has been widely attributed to a variety of functions including neuronal plasticity and neurotoxicity (Reidel, Platt & Micheau, 2003); however it is its involvement in the learning and memory process that makes it a candidate for AD therapy. For example, NMDA has been implicated in a critical memory process which occurs at a synaptic level called long term potentiating (LTP) as deactivation of the NMDAR system through antagonists has been shown to result is an inhibition of LTP in the hippocampus and cortex at the expense of cognitive function (Moahan et al. 1989). The NMDAR system has also been linked to memory impairments in a multitude of learning and memory tasks in both animals and man including those related to memory consolidation, (Kalisch, Holt, Petrovic, De Martino, Kloppel, Buchel & Dolan, 2008) spatial learning, passive avoidance, and place learning tasks (Monahan et al. 1989) most of which can be induced by introduction of NMDA antagonists into the body.

NMDA antagonists are one of the most prominent methods used to study the role of the NMDAR system in learning and memory as it enables impermanent remediation of

a critical receptor system. However, with disorders such as AD affecting a devastatingly large quantity of the population, research aimed at enhancing memory through NMDAR manipulation has also become relevant. Research intended to enhance memory, or even reverse damaged memory systems has begun looking at the effectiveness of NMDAR agonists; however overstimulation of the NMDAR system due to agonistic intervention is often more detrimental then under stimulation as it results in severe neurotoxicity (Tsai, Falk, Gunther & Coyle, 1999). Despite this, two relatively novel NMDAR agonists called, D-Cycloserine (DCS) and D-Serine (DS) have become a topic of interest among memory researchers as they have both been documented to be potent cognitive enhancers in normal subjects. Additionally, it has been demonstrated that DCS and some cases, DS can successfully reverse preexisting cognitive damage on both a molecular and structural level without the neurotoxic side effects observed with other NMDA agonists.

D-Cycloserine

D-Cycloserine (d-4-amino-3-isoxazolidone or DCS) is an antimycoacterial agent used in humans to treat nonresponsive *Mycobacterium tuberculosis* (Monohan et al, 1989). Although originally intended to combat an infectious disease, its involvement with the NMDAR system, specifically as a partial agonist, encouraged researchers to investigate its cognitive enhancing capabilities. DCS is a partial agonist on the glycine site of the NMDAR system and is therefore especially intriguing to researchers because it does not result in neurotoxicity, which is often the result of full NMDAR agonists. DCS is therefore capable of enhancing cognition without generating the detrimental effects related to neurotoxicity and in fact produces few if any side effects (Tsai et al, 1999) making it virtually ideal as a means of cognitive therapy.

In a pioneering study, Monahan et al (1989) investigated the effects of DCS on cognition in normal rats. Monahan et al. (1989) used a passive avoidance test and a place learning task to test the effects of DCS on learning and memory. The passive avoidance task was comprised of 3 consecutive days of training and involved a Y shaped runway. Each of the branches of the runway led to a separate chamber. One of the chambers was painted black while the other was painted grey so that differentiation of the two chambers would be visually possible. On day 1, the rat was placed at the bottom of the Y shaped runway and was allowed to enter one of the two chambers. A door to the chamber was then closed, trapping the rat inside. Moments later, the rat was removed and was 'forced' to enter the second chamber by default (as the door to the previously entered chamber was closed). On day 2, rats were placed at the summit of the Y runway and allowed to enter either chamber. The rat was then confined to the chamber and received a foot shock. On day 3, rats were once again placed in the Y runway where they chose a chamber to enter. Prior to testing, rats were randomly divided into a series of groups and received Intraperitoneal (IP) injections of Saline (control) or DCS (3mg/kg) either 60 min prior to the day 2 footshock, 10 seconds after the day 2 footshock or 60 minutes prior to beginning day 3 training. As a means of measuring memory, the amount of time it took the rat to enter a chamber (delay) and the chamber chosen were analyzed.

In the Place Learning Task, food deprived rats were given 3 consecutive days of training involving a standard T-maze. The T-maze consisted of a T shaped Plexiglas structure containing a food reward (cheerios) in one of the arms. Days 1-3 were used to acclimate the rats to their new environment. In order to accomplish this, rats were allowed to explore the T maze for 10 minutes with both arms of the T-maze baited with

food. On day 4, rats were given acquisition training, during which they were trained to locate the food reward that was consistently located in the right arm of the maze. In order to assure rats had learned the task, the acquisition task was continued until each rat was entering the right arm of the maze 90% of the time. On day 5 of testing, rats were trained to enter the left arm of the maze that now was home to the food reward. This portion of the test was considered a test of reversal and once again rats had to reach a minimum accuracy score of 90% to continue. Rats were injected with Saline or 3mg/kg DCS 30 minutes before training on days 4 and 5 and latency to enter the correct arm was used as a measure of memory.

The results of this study indicate that DCS improved memory function in both the passive avoidance tasks and the place learning task in rats. In the Passive Avoidance Task, this finding was signified by a significant increase in the amount of time it took rats to reenter the chamber where they had received a footshock no matter when in the course of the experiment the drug was administered. DCS was also shown to decrease latency to enter the left arm of the T-Maze during the reversal portion of the place learning task. The results of this study produced very important findings in a number of ways. First, this study effectively demonstrated that DCS can be used in normal subjects to enhance learning and memory without any obvious behavioral impairment. Furthermore, because performance on the passive avoidance task was enhanced when the drug was administered prior to footshock, it was suggested that DCS may be enhancing LTP and therefore learning capability. Additionally, the finding that rats given DCS after footshock also showed an enhanced memory for the event suggests that DCS may also be influencing consolidation of new information, therefore making the memory more

pronounced. Rats in the DCS condition that received injections 60 minutes before the retrieval task also demonstrated a cognitive advantage over their Saline treated counterparts that may suggest that DCS aids memory retrieval. While the passive avoidance task yielded results that promote the role of DCS in LTP, consolidation and retrieval, DCS was only effective on the reversal portion of the place learning task. The positive effect that DCS had on reversal of memory but lack of effect on acquisition could suggest that DCS is improving hippocampal function- a region known to be involved in learning reversal but not specifically place learning (Monahan et al. 1989). This may however, be an item of contention as other researchers have identified the hippocampus as being specifically involved in place learning (O'keefe & Nadel, 1978).

Although there is some debate over the role of the hippocampus, the results of this study do indicate that DCS does effectively enhance memory consolidation, retrieval, and tasks involving reversal of knowledge in normal animals. Furthermore, this study also produced results that provide behavioral evidence that DCS could be enhancing LTP; however, anatomical evidence is needed in order to provide additional evidence in support of this proposal.

In another study aimed at investigating the enhancing effects of DCS in normal subjects, Gabriele and Packard (2010) found evidence to suggest that DCS is able to significantly influence a hippocampus dependent function called latent extinction.

In order to simulate the effects of latent extinction in animals, Gabriele and Packard (2010) devised a food reward task during which rats learned the location of a food reward over the course of several days and then sought to extinguish the act of retrieving the food by merely exposing them to the same condition during which food

was no longer present. More specifically, during the first 9 days of the experiment, food deprived rats were placed in the start end of a straight line maze in which a food reward was located at the opposite end. Rats were trained to run to the opposite end of the maze where they were allowed to consume the reward. The amount of time it took each rat to reach the reward was recorded with shorter times suggesting stronger memory and better acquisition. Once acquisition had been achieved (day 10), rats entered the extinction phase of the experiment. During this phase, rats received a series of trials over the course of one day during which they were confined to the immediate area where the food reward had been held during acquisition; however, during this phase the reward was no longer present. Immediately following the extinction trials, rats were injected with saline or 15 mg/kg DCS. On the following day, a probe test was conducted in which rats were once again placed in the start end of the maze and allowed to run at will. As a measure of memory, latency to the goal end (which contained food during acquisition) was recorded. This was considered a test of latent extinction as opposed to response extinction because the instinct to run down the maze in order to reach the food reward was not extinguished by allowing the rat to run repeatedly to the empty goal but by mere exposure (by confinement) to the presently unbaited area without any behavioral activity.

Results of this study indicate that DCS treated rats demonstrated significant longer latency intervals to the goal end of the maze then saline treated rats. This suggests that DCS successfully enhanced latent extinction learning by improving consolidation of memory for the extinction phase. In other words, rats in the DCS condition took longer to reach the goal end because their original memory that food was present in the goal end of

the maze had been extinguished by the memory developed during the extinction phase when there was no longer food present.

While the results of studies such as the two just discussed provide evidence that DCS can be used to enhance cognition in normal subjects, many researchers focused their attention on how DCS could be used to improve or combat the effects of cognitive impairments. In one such study, Baxter, Lanthorn, Frick, Golski, Wan, and Olton (1993) successfully demonstrated that DCS may be able to attenuate normal cognitive impairments observed in the elderly. In order to explore this idea, Baxter et al. (1993) conducted two memory tasks- the place discrimination test and the repeated acquisition task, during which aged rats were the subjects.

The place discrimination task was employed as a means of testing spatial memory- a memory function that is often impaired with normal aging. This test took place over 5 consecutive days, each consisting of 6 trials. During the first 5 trials, rats were placed in the water maze from various start locations and swam freely until reaching a hidden platform located consistently across trials in the SW quadrant of the pool. The hidden platform allowed the rats to escape from the water, therefore the goal for each rat was to learn the location of the hidden platform using stationary visual cues from around the room which is thought to support spatial navigation based on the relations among distal room cues (i.e. a cognitive map of the environment). During the 6^{th} trial, a probe test was conducted during which rats swam freely in a platform-free water maze. Rats were given IP injections of saline or DCS only prior to trial 1 on each day of training and memory was scored based on how quickly the rats were able to learn the location of the

hidden platform during trails 1-5 (latency in time to reach the platform) and how much time was spent in the vicinity of the hidden platform location during the probe test.

The repeated acquisition task was designed to measure spatial memory as well as working memory which are both memory functions often times found to be disrupted as a result of aging. The repeated acquisition task was identical to the place discrimination task; however, the location of the hidden platform was changed each day in order to include a test of working memory.

The results of this study indicated that compared to young rats, aged rats performed significantly worse on both tasks used in this study; however, DCS effectively reversed the detrimental effects related to aging. More specifically, DCS treated rats' performance on both the place discrimination task and the repeated acquisition task were equivalent to those of young rats and these treated animals were able to learn the tasks quickly and adapted to the change in platform location during the repeated acquisition task. These results suggest that DCS could be used to improve both spatial and working memory and may be a realistic treatment option for the elderly who are showing deficits in their cognitive capabilities related to normal aging patterns.

While the results of Baxer et al. (1993) suggest that DCS could be used as a means of combating cognitive decline in normally aging adults, other studies suggest that DCS may also be a successful treatment for abnormal injury to the brain both at a synaptic and at a structural level. For example, Sirvio, Ekonsalo, Riekkinen, Lahtinen and Riekkinen (1992) and Fishkin, Ince, Carlezon and Dunn (1993) found that DCS can attenuate the impairing effects of muscarinic antagonists, while Schuster and Schmidt

(1992) conducted a study which found DCS is even capable of reversing the effects of hippocampal lesions in rats.

In order to investigate the potential benefits of DCS at a synaptic level, Sirvio et al. (1992) tested the effectiveness of DCS at combating the detrimental effects observed in rats treated with SCOP. SCOP is thought to primarily inhibit the highly acetylcholine dependent hippocampal region resulting in severe learning and memory impairments and is therefore often used as a model of amnesia and other types of dementia that are the result of hippocampal malfunction. In the current study, SCOP was used to impair spatial memory and was tested using the water maze.

Rats in this study were given a series of water maze trails over the course of 5 days (3 trials per day). During each trial, rats were placed in the water maze and allowed 70 seconds to locate and escape onto a stationary hidden platform located in the SW quadrant of the pool. Rats' entrance to the pool was varied across trials; however the hidden platform remained stationary throughout, therefore, memory was based primarily on spatial memory directed by external visual cues located on the walls of the testing room. Rats were treated with saline, .4 mg/kg SCOP, or .4 mg/kg SCOP with 1 mg/kg DCS 30 minutes prior to beginning each day's trials and as a means of measuring memory, escape latency to the platform and distance traveled in the pool were analyzed.

The results of this study further document the detrimental effects of SCOP, as SCOP-treated animals performed significantly worse at learning the location of the hidden platform (indicated by increased escape latency times and distance traveled). SCOP/DCS treated animals however established normal escape latency intervals and

distance traveled scores compared to controls, an indication that DCS is capable of reversing the deleterious effects of a potent muscarinic antagonist.

Fishkin et al. (1993) also conducted a study to test the beneficial effects of DCS on SCOP treated animals using a T-maze. During this experiment, rat's choice accuracy memory skills were tested over the course of 3 days, each consisting of 10 paired trials. During the 1st run of each trial, rats were placed in the bottom of the T-maze and were forced to enter one of the two arms. Once the arm had been entered, the rat was confined to the area for a matter of seconds and then placed back into the start position. During the 2^{nd} run of each trial, the rat was given a choice as to which arm to enter. Correct responses were for the rat to enter the arm that had not been entered during the 1^{st} run. If the rat responded correctly, it was confined to the arm and then began a new trial. If the rat entered the same arm as before, it was confined and received 5 brief electric shocks before beginning a new trial. Just prior to inception of each day's first trial, rats were treated with saline, l mg/kg SCOP, or 1 mg/kg SCOP and 3 mg/kg DCS.

Like in the Sirvio et al. (1992) experiment, Fishkin et al. (1993) found that SCOP-treated animals were severely impaired at this task compared to controls; however, when DCS was added, the effects of SCOP were reversed. More specifically, while rats in the SCOP condition continued to have difficulty consolidating information about how to perform the task correctly and remembering which arm had already been entered (working memory), rats treated with SCOP and DCS performed at a normal level compared to control.

Schuster and Schmidt (1992) investigated the effectiveness of DCS at improving severe brain trauma to the critically important memory structure- the hippocampus. In

this study, rats were given bilateral intrahippocampal quinolinic acid-lesions that are known to cause Alzheimer's like symptoms in rats including working memory deficits and spatial disorientation. Four days following surgery, rats treated with saline or 12 mg/kg DCS were put through a battery of memory tests aimed at determining the detrimental effects of the severe damage to the hippocampal region and the effectiveness of DCS at reversing this damage.

Over the course of 10 days (10 trials/day) rats were trained to locate a food reward within an 8-arm radial maze. Each day, the placement of the food reward changed arms and was therefore used as a test of working memory (allocentric reversal). Working memory function was calculated based on the number of double errors made during each trial. A double error was defined as a rat re-entering unbaited arms of the radial maze- an indication that they were having trouble using working memory to remember the new location of the reward and what arms had already been checked.

The results of this study show lesioned animals performing a high number of double errors compared to controls, however, animals treated with DCS showed significant improvement in their working memory function as performance was not statistically different than that of control animals. Further histologic investigation into the brains of these animals revealed no difference between DCS lesioned and control lesioned animals; therefore, Sirvio et al. (1992) suggest that DCS may be able to improve cognition by recovering remaining glutamatergic projections or reviving receptor function.

The results obtained by Sirvio et al. (1992), Fishkin et al. (1993), and Schuster and Schmidt (1992) have very important implications for the use of DCS as a possible

treatment option for diseases such as AD that affect the muscarinic receptor system and corresponding regions like the hippocampus. Investigations performed by Sirvio et el. (1992) and Fishkin et al. (1993) which focused on dysfunction at a synaptic level demonstrate that although DCS, an NMDAR agonist, does not directly increase the release of Acetylcholine, it is able to reverse the detrimental effects of neurotransmitter antagonists which inhibit ACh release. Furthermore, DCS is not only able to effectively ameliorate the resulting cognitive impairments attributed to muscarinic system failure in rats but did not cause any observable adverse side effects. Additionally Schuster and Schmidt (1993) found evidence that even the effects of severe structural damage to brain regions affected by Alzheimer's (like the hippocampus) could potentially be reversed by DCS treatment.

Based on the success observed in the animal studies conducted by Sivrio et al. (1992), Fishner et al (1993) and Schuster and Schmidt (1992) which demonstrated that DCS could effectively reverse the cognitive symptoms observed in the rat model of Alzheimer's with no observable side effects, Tsai, Falk, Gunther and Coyle (1999) began researching whether DCS could be used to combat Alzheimer's disease in humans as well. In this study, 17 participants meeting the criteria for Alzheimer's disease by the National Institute of Neurological and Communicative Disorders, Stroke, Alzheimer's Disease and Related Disorders Association and the DSM-IV were selected. Participants in the study received 4 week intervals of 50 mg/day DCS, 100 mg/day DCS and Placebo in random order (with 1 week washouts between sets) over the course of 14 weeks. In order to assess improvement due to DCS, a battery of mental tests were performed on participants at the end of each of the 4 week trials including the Alzheimer's Disease

Assessment Scale, Clinical Global Impressions of Change, and Instrumental Activities of Daily Living.

Results of this study found that participants treated with 100 mg/day DCS scored significantly better on the Alzheimer's Disease Assessment Scale than those in the Placebo or 50 mg/day DCS conditions; however, no significance was found for any of the other tests. Although DCS was not found to uniformly improve Alzheimer's patient's scores across the board, the findings of this study are still very promising and interpretation of these results provided further insight into the effectiveness of DCS.

For example, while 100 mg/day of DCS did produce some cognitive improvements, the lower dose of 50 mg/day did not which suggests higher doses of this drug are needed to generate cognitive improvements in humans. Furthermore, the results of this experiment did support the role of DCS as a cognitive enhancer in Alzheimer's patients; however, it was unsuccessful at enhancing daily living activities and the global clinical impression score. Based on these findings, it may be beneficial to use DCS for cognitive support as an adjunct with other drugs that are more specifically geared toward global functional improvement.

The results of this study were also limited in that they were based on relatively acute DCS treatment durations. Therefore, it may be appropriate to suggest that increasing duration of treatment may result in additional improvements. Another noteworthy finding that resulted from this study was that there were no side effects associated with DCS, therefore making DCS a standout treatment option among other more adverse Alzheimer's treatments.

D-Serine

Like D-Cyscloserine, D-Serine is a mediator of NMDA activity by agonistic activation of the glycine site of the NMDAR system. However, unlike DCS, DS is an endogenous ligand which works as a co-agonist on the glycine site rather than a partial agonist (Strouffer, Petri & Devan, 2003). In fact, although DS was thought to be secondary to glycine and glutamate as an agonist, abundant evidence suggests that DS is in fact more potent than glycine, leading many to believe that DS is the principle NMDAR co-agonist (Duffy, Labrie & Roder, 2007). Researchers have also discovered that DS works as a gliotransmitter, thereby allowing communication between glia and neurons (Wolosker, 2006). DS has also been defined as a mediator of excitatory synaptic transmission (Wolosker, 2006) encouraging neuronal migration and synaptic plasticity (Martineau, Baux & Jean-Pierre, 2006, Mothet et al. 2006). Furthermore, high levels of DS and corresponding NMDAR have been discovered in the hippocampus (Martineau et al, 2006) and are thought to be a primary mediator of LTP and LTD in the hippocampus (Wolosker, 2006, Duffy et al, 2007). Therefore, because it is capable of enhancing LTP and neuronal plasticity and is also important for normal NMDA and hippocampal function, DS, like DCS, has been investigated as a potential cognitive enhancer.

In a study aimed at understanding the relationship between DS and normal age related declines in cognitive function, Mothet et al. (2006) investigated DS levels in hippocampal slices of young and old animals. Close investigation revealed that the hippocampus of elderly animals contained significantly lower levels of DS. Additionally this trend was strongly correlated with a decrease in hippocampal LTP. However, saturation of the harvested hippocampal slices with exogenous DS produced increased

LTP signals in both young and old tissue samples. These results suggest that some of the learning and memory impairments caused by normal aging may be the result of a decrease in hippocampal LTP caused by a reduction in DS. Supplementation of DS into the brains of DS deprived animals however, may be an effective means of enhancing LTP in the hippocampus and thereby enhancing learning and memory.

Additionally, Yang, Qiao, Wen, Zhou, and Zhang (2004) found that in the SAMP8 mouse, LTP in the hippocampus significantly decreases with age. However, exogenous treatment of DS effectively reversed this decline. Due to a variety of genetic ailments such as increased expression of A-B, amyloid precursor protein, and a loss of NMDAR and cholinergic function (Yang et al., 2004), the SAMP8 mouse is often used as model of aging and rapid cognitive decline. Therefore, Yang et al. (2004) sought to determine whether LTP in hippocampus of the SAMP8 mouse was normal. By measuring electric recordings of SAMP8 hippocampal slices at 2, 6 and 12 months of age, it was determined that compared to control, LTP in the hippocampus was severely diminished especially at the 12 month time point. Next, Yang et al. (2004) treated 12 month old SAMP8 hippocampal slices with exogenous DS and found a significant increase in LTP. Taken together, the research conducted by Mothet et al. (2006) and Yang et al. (2004) demonstrate that some of the cognitive decline observed in the aged, occurs as the result of a decrease in LTP in the hippocampus. Additionally, DS is capable of enhancing LTP and could therefore be of potential use as a therapy for age related cognitive detriments.

Behavioral studies have also suggested that DS can be used to reverse memory related impairments. For example, Andersen and Pouzet (2004) treated 7, 9, and 11 day old rat pups with an NMDA antagonist, phencyclidine (PCP), to establish a model of

long-term cognitive dysfunction. The Morris water maze was used to test spatial reference memory, spatial reversal learning, and spatial working memory in the same rats at 55-70 days old. Thirty minutes prior to beginning each test day, rats were treated with either 640 mg/kg or 1280 mg/kg doses of DS. The spatial reference task was conducted over 4 days and was used as a measure of memory acquisition. During this test, rats were given 4 trials per day during which they were allowed 60 seconds to escape onto a submerged platform located consistently in the N quadrant of the water maze throughout the duration of trials. Two days after the reference memory task, the spatial reversal learning task was started. The procedure for the reversal memory task was identical to that of the spatial reference memory task, however, the hidden platform was relocated to the S quadrant of the water maze, therefore forcing rats to abandon a previously developed memory in place of a new pattern.

Results of this study suggest that antagonizing the NMDAR system early in life results in long-term impairments in the ability to perform spatial memory tasks. More specifically, PCP-treated animals performed poorly during the reference memory task and also demonstrated significant deficits in learning during the spatial reversal task. This suggests improper spatial memory function. Additionally, Andersen and Pouzet (2003) noted a pattern in swim behavior with PCP treated animals which consistently demonstrated an idiocentric instead of allocentric search strategy. Idiocentric patterns, thought to be mediated by the striatum (Andersen & Pouzet, 2003), are those in which the subject locates an object using the relationship between its own body position and space. This strategy is considered impaired because while it demonstrates some learning, it is less productive and more time consuming than other strategies. On the contrary

allocentric search patterns are indicative of normal spatial navigation and are mediated by the hippocampus. Therefore, PCP-treated animals were demonstrating a deficit in hippocampal function but normal striatal function. When results of the DS-treated animals were analyzed, it was discovered that PCP animals treated with DS did not demonstrate improvements on the reversal task; however, they performed consistently at normal levels during the spatial reference memory. Furthermore, it was discovered that DS-treated animals began using the same allocentric search patterns observed in normal animals. This shift from using the less accurate and more time consuming method of idiocentric searching to a normal allocentric search pattern suggests that DS may be improving hippocampal function.

Although DS has been implicated as a mediator of normal hippocampal function, some research has suggested that DS could be acting on another memory system- the striatum. A study conducted by Stouffer, Petri and Devan (2004) sought to determine whether administration of DS would enhance spatial memory in rats during water maze tasks. To test this, the delayed match-to-place task was implemented and different memory system measures were accumulated for rats treated with I.P. injections of either DS or a control substance. The delayed match-to-place (DMP) task involves 4 trials per day over 6 days of water maze training. Each day, rats were subjected to 3 trials in which they were placed on a sinking platform (allowing them 3-5 second to orient themselves) which was located in the same position throughout the experiment. Rats were then given 60 seconds to locate a hidden platform submerged 1 cm below the water line. Two hours after completing the 3rd DMP task of the day, rats were given a 4th trial in which they entered the pool from a new location. The same procedure was followed on all 6 days of

testing with the exception that the location of the hidden platform was different on each day of testing. Although, DS did not have a significant effect on escape latency, heading error or cumulative distance swum; DS treated animals spent significantly more time searching the quadrant where the platform had been located on the previous day compared to their saline injected counterparts. This DS induced alteration in win-stay behavior indicates that DS is in fact regulating learning patterns and may be primarily influencing the striatum, a habit-based learning system which has been linked to win-stay as opposed to win-shift behavior (Stouffer et al., 2004).

Based on the research just discussed, DS appears to be a very capable and realistic form of cognitive therapy for patients suffering from AD or other cognitive disorders related to the hippocampus. *In vivo* studies have demonstrated with certainty that DS is critical for LTP in the hippocampus to occur (Mothet et al., 2006, Yang et al., 2004) and behavioral research suggests that DS can attenuate the hippocampal impairments that result from exposure to NMDA antagonists during development (Andersen & Pouzet, 2004). With an interruption in normal hippocampal function believed to be one of the main causes of memory impairments in AD patients, it seems reasonable to suggest that DS could offer some potential advantages for AD patients; however, many advise otherwise. Stouffer et al. (2004) suggest that DS may be primarily influencing the striatum, leaving the hippocampal system in failure while other researchers propose that DS could, in fact, cause more harm than good. For example, Butterfield and Boyd-Kimball (2004) point out that one of the primary insults observed in AD, the amyloid-B-peptide, causes neurotoxicity by increasing levels of DS, thereby over-stimulating the NMDA system. Furthermore, increased levels of Serine Raispase, the precursor of DS,

have been discovered in the postmortem hippocampal specimens and an overall increase in NMDAR activity in the brains of AD patients have been reported (Lipton, 2004, Scolari & Acosta, 2006).

The discussion of D-Cycloserine and D-Serine is an interesting one. Both DCS and DS work by activating the NMDAR system; however their differences are of great importance too. DS, an endogenous NMDA ligand was once believed to be secondary to glycine but is now regarded as the primary agonist on the glycine site of the NMDAR system. Lack of DS results in severe malfunction of the hippocampus, a decrease in neuronal plasticity and LTP without which learning and memory are not possible. Introduction of DS into depleted systems has been shown to improve cognition and hippocampal function markedly; however, over-activation of the NMDAR system due to an overabundance of DS can be devastating. DCS, on the other hand, is not endogenous. Created to treat resistant forms of tuberculosis, DCS is not naturally present in the brain and when artificially administered works only as a partial agonist on the NMDAR glycine site. Research aimed at investigating the potential of DCS as a cognitive therapy has demonstrated that it is not only capable of improving working memory, spatial memory, (Sirvio et al. 1992, Fishkin et al. 1993, Baxter et al. 1993) consolidation, retrieval, reversal (Monahan et al. 1989) and extinction learning (Gabriele and Packard 2010) in normally functioning mammals but has also been documented to improve the effects of cognitive impairments at a molecular (Sirvio et al. 1992 and Fishkin et al. 1993) and structural level in both animals (Schuster and Schmidt 1992) and man (Tsai et al., 1999). Of possibly more importance, is the fact that DCS has not been documented to cause any side effects including those related to neurotoxicity. The result of being a mere

partial agonist, DCS lacks the potency of DS but may in fact be more beneficial given the lack of side effects.

The Present Study

The present study sought to investigate the potential benefits of DCS and DS in a SCOP-induced model of AD. Using the competitive place task version of the Morris water maze, the cognitive benefits of DS and DCS were investigated on a number of levels. The primary focus of the study was to determine whether DS and DCS are capable of attenuating the impairing effects of SCOP. DS and DCS were also compared in order to establish whether one is advantageous over the other. Additionally, the competitive place task was designed in order to test hippocampal and striatal function independently. Because there is some debate as to whether DS is affecting the hippocampus or the striatum, using the competitive place task provided further evidence as to which system this drug is primarily influencing.

In order to accomplish these goals; rats were randomly divided into 6 conditions (Saline, SCOP, SCOP/DS, SCOP/DCS, DS, DCS). Rats were then subjected to a version of the Morris Water Maze known as the Competitive Place Task (CPT) (McDonald, Hong, Craig, Holahan, Louis, and Muller, 2005). This particular task was implemented over the course of 8 days. On days 1-6 untreated rats were taught to learn the location of a hidden platform (old location). On day 7, rats were administered an I.P. injection of Saline (SAL), 1 mg/kg Scopolamine (SCOP), 1 mg/kg Scopolamine + 100 mg/kg D-Serine (SCOP/DS), 1 mg/kg Scopolamine + 1 mg/kg D-Cycloserine (SCOP/DCS), 100 mg/kg D-Serine (DS), or 1 mg/kg D-Cycloserine (DCS) 60 minutes prior to testing. In order to assess working and spatial memory performance, rats then underwent a mass

learning trial during which they were required to learn a new hidden platform location (new location). 24 hours after the conclusion of the last mass training trial, a probe test was conducted during which the hidden platform was removed from the pool entirely and the free swim methods of the rats were analyzed as a means of determining memory strength, consolidation and recall of the previously learned platform placement. The competitive place task was chosen because it employs several aspects of learning and memory including the ability to acquire and learn short-term spatial information as well as long term memory consolidation. Furthermore, the CPT was designed to test 2 types of memory, spatial navigation and habit-based memory, independently (McDonald et al., 2005). More specifically, the first 6 days of the experiment, during which the rats were taught to learn the location of a stationary hidden platform over the course of several days, was used as a means of testing habit based memory, which has been suggested to be mediated by the striatal system (Turchi et al., 2008). On the day of drug treatment however, rats are likely using spatial navigation and working memory, regulated by the hippocampal system (Herrera-Morales, Mar, Serrano & Bermudez-Rattoni, 2007), to learn the new location of the platform. This switch in memory system involvement occurs because while engaging in multiple trials per day over the course of several days is likely to result in the development of a habit-like memory for the location of the platform, leaning the position of a platform on single day of testing is not. Instead, a single day of testing causes subjects to rely on using stationary visual objects for direction and working memory enables for the accumulation of knowledge regarding platform placement. Therefore, the design of the CPT is considered a test of multiple memory systems.

As a means of measuring memory strength for previous events, escape latency to the hidden platform was evaluated on days 1-7 of the study and on the day of the probe test the amount of time spent in each of the pools quadrants was quantified.

The hypothesis of the present study is multidimensional. First, based on previous research involving SCOP, DCS and DS, it was hypothesized that SCOP-treated animals would show significant deficits in acquisition of new information on the day of drug treatment. It was therefore predicted that on the day of drug injection, rats treated with SCOP would demonstrate significantly longer escape latencies to the hidden platform compared to control. This would indicate a decrease in the ability to use working and spatial navigation to learn the new location of the platform- a suggestion that the hippocampus is not functioning properly. On the day of the probe test, SCOP-treated animals would also show a preference for the location of the platform location learned during the first 6 days of training when memory was habit-based (old location). Results reflecting this trend would suggest a strong habit-based memory, and therefore, an intact striatal system (Turchi et al., 2008; McDonald et al., 2005).

For rats in the SCOP + DS and SCOP + DCS treatment groups, it was hypothesized that there would be no observable deficits, demonstrating escape latencies and quadrant preferences statistically no different than those of control. Results consistent with this hypothesis would suggest the efficacy of DS and DCS to attenuate the impairing effects of SCOP. Although it was predicted that DS and DCS would be successful at ameliorating the effects of SCOP, a more detailed interpretation of the data may suggest some difference between the drugs. For example, the literature suggests that DCS is primarily influencing the hippocampus, while DS research has conflicted as to whether it

is affecting the hippocampus or the striatum. Therefore, although it is only a partial agonist, it was hypothesized that DCS might demonstrate an advantage because it is targeting SCOP more actively then DS. If this suggestion is correct then rats treated with DCS would exhibit a preference for the new location when probe tested compared to DS-treated animals. On the other hand, DS-treated animals would favor the old location if DS is enhancing striatal function as opposed to the hippocampus.

For the DS and DCS conditions, it was hypothesized that animals in these groups would show a cognitive advantage over all other groups. This advantage might be demonstrated by quicker, more accurate learning curves on the day of drug injection. Because both DS and DCS have been linked to an increase in LTP in key memory regions, animals in these conditions should perform significantly better than even the control group. Past research has identified both DS and DCS as being cognitive enhancers; however, there is debate as to whether one drug is more effective than the other. Therefore, this study was designed to further investigate the cognitive enhancing capabilities of each of these drugs independently and in comparison.

If the hypotheses proposed in the current study are correct, this study will provide additional evidence that DS and DCS are potential cognitive enhancers and may help ease the dispute as to whether DS is primarily affecting the hippocampus or the striatum. Additionally, the results of this study could be an important addition to AD research as they would suggest that NMDA agonists could reverse some of the cognitive detriments observed in AD.

Method

Subjects

63 male Long-Evans hooded rats from Charles River colonies were used for the purposes of this study. Animals were individually housed and remained on a consistent 12 hour light: dark cycle for the entirety of the experiment. Food and water were available to animals at all times. Animals were given at least 5 days to acclimate to their new environment before water maze trials began. Rats were handled daily for 3 days prior to inception of this study in order to alleviate handling as a stressor.

Materials- The Water Maze

The water maze consisted of a large circular pool of water approximately 6 ft in diameter and 60 cm deep. The pool was filled with water which was maintained at a temperature that ranged from 21-25 degrees Celsius. During trials in which a hidden platform was present, a 12x12 clear Plexiglas platform was submerged 1 cm below the surface of the water so that it was not visible to the animal. To further ensure that the platform was completely hidden, white nontoxic tempera paint was added to the water in order to make the water color opaque. A video camera with accompanying VHS recorder and tracking device was used in order to obtain accurate and reliable measures of escape latency and quadrant preference during each trial. Stationary visual cues such as a poster, table and chair were used as spatial navigation markers.

Procedure

Based on the suggestions of previous animal research, it was determined that subjects for each group would consist of 10 or 11 male Long Evans rats for the Control and SCOP groups while 13 male Long Evans rats were used for the SCOP + DS and

SCOP + DCS groups. Due to time limitations, the DCS and DS groups consisted of 8 rats each. For the purpose of this study, rats were run in 3 sets of 2 groups. The Saline (control) and SCOP groups were run during set 1, SCOP + DS and DS alone groups were run during set 2 and SCOP + DCS and DCS alone groups were run in set 3. Experimenters were blinded to the drug group and animals from each group were staggered in order to prevent bias. A version of the Morris Water maze called the Competitive Place Task (CPT) (McDonald et al. 2005) was used in order to assess learning and memory. The CPT was chosen over the original Morris water maze because it enables researchers to not only study the ability to use working memory, spatial memory, consolidation and recall but also provides the opportunity to investigate competing memory systems. The CPT is made up of 3 distinct phases and was carried out over 8 days.

Phase 1

Phase 1 of the competitive place task consisted of 6 days of extensive water maze training. On each of the 6 days, untreated rats were given 4 trials during which they learned the location of a hidden platform located in the South West (SW) quadrant of the pool. During each trial, the rat was placed into the water maze facing the wall from various points of entry. Rats were allowed 60 seconds to locate the hidden platform within the maze. Once the rat found and escaped onto the hidden platform, it was given 30 seconds to remain on the platform (and out of the water) at which point the animal was returned to its home cage. If the rat was unable to locate the hidden platform within allotted 60 seconds, it was gently guided to the platform by the researcher and was then given its allocated 30 seconds of escape time. Although rats were placed in the pool from

a different location at the beginning of each trial, the hidden platform remained stationary (in the SW quadrant, also referred to as the 'old location') throughout phase 1. Phase 1 of the experiment was designed to measure two things. First, because the animals were untreated during this phase, this allowed for the quantification of a baseline learning rate which was used to ensure no pre-existing differences between groups. Second, this prolonged training phase, during which rats repeatedly swam to the same location over the course of multiple trials for several days provided a form learning and memory thought to be primarily habit based and therefore regulated by the striatum.

Phase 2

On day 7 of the experiment, phase 2 of water maze training began. On this day, rats were given I.P. (Intraperitoneal) injections of Saline, 1mg/kg SCOP, 1 mg/kg SCOP + 100 mg/kg DS, 1 mg/kg SCOP + 1 mg/kg DCS, 100 mg/kg DS or 1 mg/kg DCS depending on assigned group. Drug doses were determined based on previous research in which these specific drugs were used to inhibit or enhance performance on memory tasks without resulting in motor impairment or neurologic toxicity. Approximately 1 hour after I.P. administration of drugs, rats began a series of 8 trials within 1 hour. During phase 2, the hidden platform was relocated to the North East (NE) quadrant of the pool (new location) but remained in this location for all 8 trials. Once again, rats were placed from varying points of entry into the water maze facing the wall. Rats were allowed 60 seconds to reach and escape onto the hidden platform. If they did not find the platform within the allowed 60 seconds they were gently guided to it by hand. They were then given 30 seconds to rest on the platform before being returned to their home cage to await the next trial. This phase of the experiment allowed for the measure of the ability to learn the

location of a hidden platform while under the influence of these various drugs. Because all trials in phase 2 took place over the course of an hour on the same day, this part of the experiment focused on spatial learning and working memory (mediated primarily by the hippocampus) as opposed to habit based learning which was the focus of phase 1.

Phase 3

In order to ensure complete wash out of drugs, phase 3 (day 8) of the CPT began 24 hours after completion of phase 2. During this phase, a single probe test trial was conducted. During this trial, the hidden platform was removed from the pool. Rats were placed in the water maze facing the wall from one consistent location in the North West quadrant of the pool and allowed 60 seconds to swim freely. During the probe test, the amount of time the rat spent in each individual quadrant was quantified. Quadrant preference has been proven as a reliable measure of memory strength (Morris, 1984) and as such was used in this experiment as an indicator of memory recall and consolidation of previously learned information.

Results

A 6 x 2 mixed ANOVA (Group: Saline, SCOP, SCOP + DS, SCOP + DCS, DS, DCS; Day: average escape latency Days 1-3, average escape latency Days 4-6) was conducted to eliminate pre-existing group differences. There was no significant main effect or interaction found between groups during phase 1 of the experiment (See figure 1).

For phase 2 of the experiment, a 6x2 (Group x Block) mixed ANOVA (Group: Saline, SCOP, SCOP + DS, SCOP + DCS, DS, DCS; Block: average escape latency Trials 1-4, average escape latency Trials 5-8) was conducted in order to analyze the

enhancing or inhibiting properties of these drugs on spatial memory function. Results indicated a significant main effect of Group, $F(5, 57)=5.429$, $P<001$. Further analysis revealed that there was a significant difference in escape latency between Saline and SCOP ($p<.01$), Saline and SCOP + DS ($p<.01$), SCOP and DCS ($P<.05$), and between SCOP + DS and DCS ($p<.05$). There were no other significant differences between groups (See Figure 2). No other significant main effects or interactions were found for results obtained during phase 2 of the experiment.

In order to measure overall quadrant preference on the day of the probe test, individual analyses were run. A 2 x 2 (Group x Quadrant) mixed ANOVA with repeated measures on the second factors (Group: Saline and SCOP; Quadrant: Old and New) was performed .Results indicated a significant crossover interaction between group and quadrant, $F(1,38)=15.69$, $P<.001$. (See Figure 3 and 8).

A second 2 x 2 (Group x Quadrant) mixed ANOVA repeated measures on the second factor in which group included Saline and SCOP + DS and Quadrant was Old and New location was conducted. Resulted indicated a main effect of quadrant $F(1, 44)=18.63$, $P<.001$ and a significant interaction $F(1, 44)=82.169$, $P<.001$ (See Figure 4 and 8).

A third 2 X 2 (Group x Quadrant) mixed ANOVA with repeated measures on the second factors (group: Saline and SCOP + DCS; Quadrant: New and Old) was performed. The results revealed a main effect of quadrant $F(1, 44)=5.194$, $P<.05$ and a significant interaction of group and quadrant $F(1, 44)=41.972$, $p<.001$ (See Figure 5 and 8).

A fourth 2 X 2 (Group x Quadrant) mixed ANOVA with repeated measures on the second factors (group: Saline and DCS; Quadrant: New and Old) was then performed. This test revealed a significant interaction $F(1, 34)=6.709$, $p<.05$ (See Figure 6 and 8).

Discussion

The objective of the present study was to investigate whether DS and DCS are capable of attenuating the memory impairments observed in a SCOP induced model of AD. In order to address this question, several aspects regarding the impact of DS and DCS treatment were considered. For example, some research has suggested that DCS is primarily influencing the hippocampus; however, results obtained by Stouffer et al. (2004) indicate that DS may have a stronger influence on functions controlled primarily by the striatum compared to the hippocampus. Conflicting interpretations of data have led to uncertainty as to which memory system DS is primarily influencing. In order to address this, a water maze task was used which was designed to test spatial/working memory and habit memory function independently (McDonald et al., 2005). Because spatial and working memory have been linked to hippocampal function and habit based memory is thought to be mediated by the striatal system, this design allowed for a more in depth look at what system DS is targeting. The investigation into system influence then leads to another question regarding efficacy. Despite DS and DCS sharing similarities as mediators of the NMDAR system, if DS does in fact have a more potent affinity for the striatum then it seems reasonable to suggest that DCS could offer a greater advantage when attempting to correct hippocampal dysfunction. While there have been experiments designed to study the effects of these two drugs independently, there are few which have made direct comparisons of effectiveness. Therefore, the capability of these drugs to

enhance learning and memory in both disabled and normal rats was also assessed in order to make a direct comparison of benefit.

The first hypothesis of this study suggested that SCOP could be used as a model of cognitive dysfunction, making it difficult for new spatial memory to be obtained. Therefore, it was predicted that rats treated with SCOP would show increased escape latencies compared to control suggesting a deficit in working and spatial memory. It was further hypothesized that DS and DCS would attenuate the impairing effects of SCOP, making learning and memory behavior similar to that of control. However, if DS is primarily influencing the striatum than DCS may be a superior means of attenuating SCOP as it is targeting the hippocampus more directly. It was also predicted that when given alone, DS and DCS would enhance learning and memory and therefore rats in these groups would demonstrate a cognitive advantage over control.

Results of this experiment demonstrated that SCOP treated animals achieved consistently longer escape latencies to the location of the platform during phase 2 of the CPT (drug day) compared to control; however, average swim speed was not statistically different than control, suggesting the possibility that these findings are not be the result of a drug induced motor impairment. Further analysis illustrated that escape latencies for animals treated with SCOP + DS were not significantly different than rats treated with SCOP alone and performance of rats in DS and DCS groups were statistically similar to those of control. However, rats which were treated with SCOP + DCS exhibited a significant improvement in function, making them statistically no different from the group treated with Saline.

The results of this portion of the study are important and informative in many ways. First, rats in the SCOP condition did show the predicted memory impairments. Rats in this condition were exposed to a series of trials during which they had consistently longer escape latency times compared to control, an indication that the ability to acquire new memory was diminished. Because phase 2 was designed to test spatial and working memory and therefore hippocampal function, this finding suggests that SCOP was effective at impairing hippocampal function, resulting in an inability of animals to use spatial and working memory while learning the new location of the hidden platform. This result is likely due to the fact that SCOP was depleting the hippocampus of ACh, rendering it disabled.

Rats which were treated with SCOP + DCS demonstrated a significant improvement in function, making their performance statistically no different than that of control. This phase of the CPT was designed to test hippocampal function through working and spatial memory performance. Therefore, the resulting improvement observed in animals treated with SCOP + DCS suggests that DCS is effectively targeting, activating and improving the impairment to the hippocampus caused by SCOP. This attenuation of SCOP induced memory failure demonstrates that DCS may be an effective means of treating memory impairments, specifically those related to the hippocampus, and perhaps some cognitive symptoms related to AD.

The lack of significant findings involving the SCOP + DS group is also of interest. First, the fact that there was no significant improvement in memory function observed in the SCOP + DS group indicates that 100 mg/kg DS is not capable of attenuating SCOP impairments in this memory test. Although past research has

demonstrated that DS is able to improve cognitive impairments, the results of this study suggest that DS may not be an effective means of treating AD or other disorders related to hippocampal malfunction. Furthermore, the lack of improvement to hippocampal function through administration of DS may lend further support to the suggestion that the primary effect of DS may not be on the hippocampus but rather on the striatum (Stouffer et al., 2004).

The results of this study also indicate that DS and DCS alone are not effective at improving normal hippocampal function. In other words, because the DS and DCS groups performed at the same statistical rate as control, there is no indication that DS and DCS alone resulted in improved hippocampal function and therefore working or spatial memory in uninhibited rats.

Phase 3 of the experiment, during which animals completed a single probe test trial, was designed to evaluate memory strength for previously learned hidden platform locations. This was investigated by quantifying the amount of time spent in each of the quadrants, with greater amounts of time indicative of increased memory strength. The CPT was designed to evaluate hippocampal and striatal function independently by encouraging formation of a habit-based memory for a hidden platform located in the SW quadrant of the pool (old location) during phase 1 of the experiment followed by development of a spatial/working memory for a hidden platform located in the NE quadrant (new location) during phase 2. Because it is suggested that habit-based memory is linked to striatal function (Turchi et al., 2008) while spatial/working memory is related to hippocampal function (Herrera-Morales et al., 2007), the CPT allowed for the study of competing memory systems and their influence on behavior by evaluating the quadrant

preference of the animal. A preference for the old location would indicate a dominant habit-based memory, controlled by the striatum while a new location preference would be indicative of superior hippocampal involvement and resulting spatial/working memory function (McDonald et al., 2005).

For this portion of the study it was hypothesized that animals with normal or heightened hippocampal function would demonstrate a preference for the new location quadrant while rats with malfunctioning hippocampal function or enhanced striatal function on drug day would prefer the old location quadrant. More specifically it was predicated that SCOP treated animals, whose hippocampus had been inhibited during phase 2, would demonstrate this affect by spending more time in the old location quadrant during the probe test. The suggestion that DS may be primarily enhancing the striatum led to the prediction that animals treated with DS would also show a preference for the old location during probe test. Rats in the control and DCS groups, which were predicted to experience normal (control) or enhanced (DCS) hippocampal function during phase 2, would then demonstrate a spatial preference for the new location quadrant throughout the probe run.

The results of the probe test indicated that while rats in the control condition exhibited a normal and significant preference for the new location, rats treated with SCOP spent significantly more time searching the old location of the pool. Rats in the SCOP + DS condition showed a significant preference for the old location that was even more robust than that of the SCOP group, while animals in the SCOP + DCS condition demonstrated a significant preference for the old location compared to control that was not different than that of SCOP. DS alone resulted in an equal preference for the old and

the new location. DCS alone also resulted in a significant preference for the old location compared to control but a lack of significant preference when compared alone.

The results produced by this portion of the experiment offer several implications. SCOP treated animals did perform as expected, spending most of the time searching the old location. This suggests that a lack of hippocampal involvement throughout phase 2 of the experiment made it difficult for rats in this condition to obtain and consolidate new spatial memory. A preference for the old location suggests that SCOP treated animals did not develop a strong memory for the new platform location, despite being most recently learned; however, an enhanced preference to search the old platform location suggests a functioning system related to the habit-based memory created by the striatum during phase 1.

The amplified preference for the old location observed in the SCOP + DS group is of significant importance. Like animals in the SCOP condition this old location preference suggests a stronger habit-based memory for the old location developed during phase 1 than the spatial memory created during phase 2. The significant preference for the old location compared to even the SCOP group suggests not just a normally functioning striatum but an abnormally enhanced one, resulting in an even greater habit-based memory. This finding could be explained by exploring the competitive nature of the hippocampal and striatal memory systems combined with the influence of DS. Based on some competitive memory systems theorists, the hippocampus and the striatum are in constant competition for influence (Lee, Duman, and Pittenger, 2008). The hippocampus; however, is the dominant system making striatal memory function less influential. When a drug such as SCOP, which inhibits the hippocampus, is administered the striatum

becomes the more prominent system. That is to say that without competition from the hippocampus, the function of the striatum becomes the primary contributor to recall, leading, in this case, to an abnormally strong memory for the habit based memory learned during phase 1 for the old location. In addition to this surge in striatal function due to a lack of competition from the hippocampus, DS may have been providing additional aid to the striatum making the habit-based memory for the old location especially strong in rats treated with both SCOP and DS. This finding not only suggests that there is competition between systems but also lends further support to the notion that DS is in fact a potent enhancer of the striatum and not the hippocampus.

The results of the DS alone group offer additional support for both the competitive memory systems hypothesis as well as the theory that DS is primarily influencing the striatum. This can be interpreted by looking at the behavior of rats in this groups which demonstrated an equal preference for both the old and new platform locations. Although in control animals, normal hippocampal function resulted in a predominant memory for the new location, the artificial enhancement of the striatal system through the introduction of DS may have resulted in a balanced memory for both locations. Rats in this condition no longer appear to have a dominant and submissive memory system but rather two equally influential regions resulting in equivalent memories for both the habit (old location) and spatial (new location) components.

On the day of probe, SCOP + DCS treated animals showed memory patterns similar to that of the SCOP-treated animals, demonstrating a significant preference for the old location compared to controls. These results were unexpected and may suggest that although DCS did attenuate the impairing effects of SCOP on the day of

administration, it was unable to remediate the long term effects of SCOP. Another explanation for this finding could be developed by reviewing the results obtained by Monahan et al. (1989) who found that when administered after footshock, DCS significantly enhanced memory consolidation. Therefore, it could be possible that the introduction of DCS following phase 1 resulted in enhanced consolidation of what was learned during the days prior to drug administration. This enhancement in consolidation would then make the memory for the old location the primary memory recalled.

When a within group comparison was conducted, the DCS alone treatment group showed no significant preference for either the old or the new location; however compared to control they showed a significant preference for the old location. This finding did not match the proposed hypothesis and the interpretation of these results is not clear. The enhanced preference for the old location compared to control could suggest that like DS, DCS is influencing the striatum, strengthening the habit based memory controlled by it. Or, as suggested with the SCOP + DCS group, it may be possible that DCS is enhancing consolidation of memories developed prior to administration rather than bolstering new memory formation. The lack of significance when the DCS group's quadrant preference was compared against itself may also suggest increased memory strength for the old location due to positive intervention of the striatum or because of increased consolidation for memories learned during phase 1. Although postulations can be made with regard to these findings, there is no clear explanation and as such, the subsequent effects of DCS treatment need to be further investigated.

The purpose of the present study touches on many areas within the scientific field. First, with little sound advancement in AD treatment, it is important to continue to

investigate drugs which have the potential to reverse or even just lessen the cognitive decline associated with AD. Although the NMDA receptor system is accepted as being a critical component in learning and memory, LTP and neuronal plasticity (Bliss & Collingridge, 1993; Rezvani, 2006), its use as a cognitive aid has come into question due to side effects such as neuronal toxicity (Wolosker, 2006). Regardless, past research has found evidence that DS treatment can attenuate memory impairments in an array of experimental settings. DS, a gliotransmitter and partial agonist on the Glycine site of the NMDA receptor encourages synaptic plasticity and cell migration and is thought to be a primary mediator of LTP and LTD, all of which are critical for normal memory function. DS has been found to reverse cognitive decline in normally (Mothet et al., 2000) and artificially aged rats (Yang et al., 2005) and improve memory performance in both impaired (Andersen and Pouzet, 2004) and normal animals (Stouffer et al., 2004). Despite these findings, the current investigation found that DS did not result in significant reversal of hippocampal damage but did provide evidence to suggest that DS is primarily influencing the striatum as was found by Stouffer et al. (2004). This conflict in findings may be explained by looking more closely at the ways in which DS has been previously investigated. For example, in the case of Mothet et al. (2000) and Yang et al. (2005), the striatum was not considered at all, but rather, *in vivo* hippocampal slices were saturated with exogenous DS. Had both striatal and hippocampal slices been subjected to DS treatment, the results may have suggested that although DS is capable of activating the hippocampus, it has a natural affinity for the striatum which is more potent than that of the hippocampus. The behavioral study conducted by Andersen and Pouzet (2004) may serve as a better model of comparison for the current study as both were designed to

observe water maze behavior in order to investigate memory function in impaired animals. Andersen and Pouzet (2004) however, used doses of DS over 10 times greater than what was used in the present study. The large amount of DS used by Andersen and Pouzet (2004) may have been substantial enough to fully saturate the striatum and still leave enough excess DS in the system to also enhance hippocampal function. The competitive nature of these systems (Lee et al., 2008) would have then made the influence of the hippocampus more obvious to observers than that of the striatum.

Like DS, DCS influences NMDA function by working as an agonist on the glycine site of the NMDA receptor; however, unlike DS, which is a co-agonist, DCS is merely a partial agonist and is therefore unable to activate the NMDAR system with the same propensity as DS. This feature; however, should not be seen as a disadvantage as DCS has been shown to be capable of positively influencing learning and memory but unlike DS it has not been linked to neurotoxicity which results from an overly active NMDA receptor system. Research has suggested that DCS is capable of enhancing LTP, consolidation, retrieval (Monahan, 1989), and latent extinction (Garbriel and Packard, 2007) in normal rats. DCS has been documented to reverse neurologic impairment caused by SCOP in the T-Maze and water maze (Sirvio et al, 1992 and Fishkin et al. 1993) and has been shown to reverse working memory impairments in hippocampal lesioned rats (Schuster and Schmidt, 1992). Additionally, in the case of Tsai et al. (1999) DCS has been proven a viable treatment option for cognitive dysfunction in human AD patients. The results of the current study do support many of these findings and suggest that administration of DCS in SCOP-treated animals results in an enhancement of spatial and working memory making performance significantly no different than control. However,

this positive influence on hippocampal dysfunction was not found once drug washout had occurred, resulting in behavior and memory preference similar to that of the impaired group. Furthermore, the results of the present study did not support the findings of Monahan et al. (1989) and Gabriele and Packard (2007) who described DCS as being able to significantly enhance learning and memory in the normal subjects. Instead in the current study, DCS alone did not result in any cognitive benefit to working memory, spatial memory, recall or consolidation of memory in normal subjects. Despite this, these results suggest that compared to DS, DCS is a superior means of correcting learning and memory dysfunction related to hippocampal failure and may possess properties that make it an ideal treatment option for diseases such as AD. The inability of DCS to attenuate the effects of SCOP the day following drug administration may suggest that while DCS is capable of attenuating hippocampal memory ailments, its influence is acute and it must be present in the system in order to produce a positive effect.

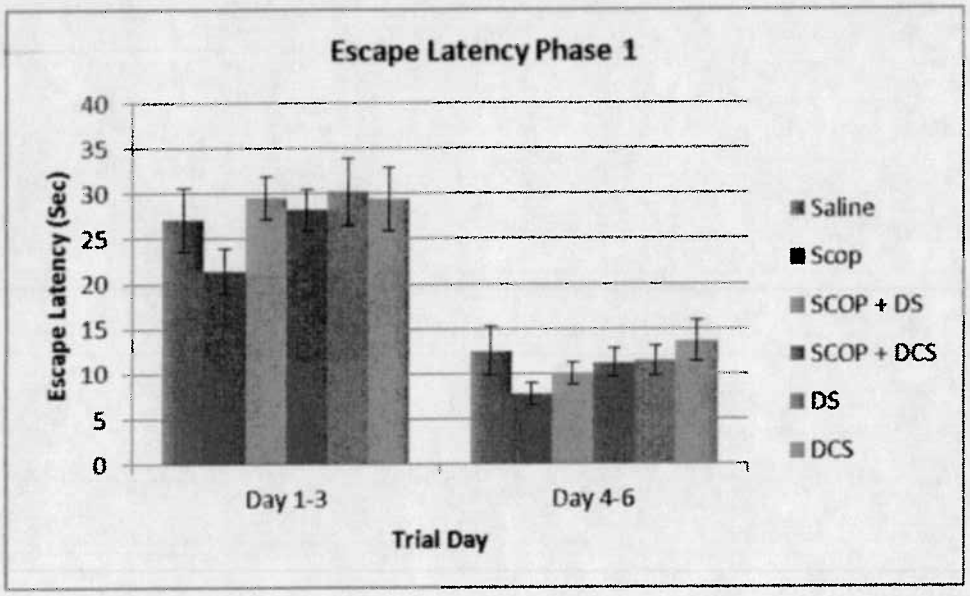
The present study was developed in order to investigate the benefits of DS and DCS in greater depth. An experiment was designed in order to examine the capabilities of these drugs to correct the learning and memory impairments observed in the SCOP model of AD. Additionally, DS and DCS were studied in comparison in order to determine system influence and effectiveness in both impaired and normal subjects. By using SCOP to deplete the hippocampus of ACh, we were able to create a model of cognitive dysfunction in which rats not only showed a significant deficit in working and spatial memory function but also demonstrated an inability to consolidate and recall newly learned material on a later date.

Using this model of cognitive dysfunction allowed for the study DS and DCS as potential treatment options for disorders such as AD which devastate the hippocampus and cause learning and memory impairments. Based on the results of the current study, it seems that further investigation into the potential benefits of these drugs is important and offer several implications for future research. In terms of DS the lack of positive manipulation to hippocampal function in both normal and impaired animals should not imply that DS is an ineffective cognitive enhancer but rather that it may be more beneficial to the striatal system. Therefore it may be of interest to begin testing the effects of DS on subjects with impairments to the striatum rather than the hippocampus.

Additional research on DCS should also be conducted and shown more consideration in realm of AD research. DCS has been documented to benefit cognition and learning in many experimental studies and has even been effectively used in the human population to treat AD (Tsai et al., 1999). However, despite consistent evidence suggesting that DCS is a potent and capable cognitive enhancer, little research on DCS has been conducted in the last 10 years. Although the current study did not support the notion that DCS provides a cognitive advantage to those in the normal population, DCS treated animals that received SCOP did exhibit an improvement in working and spatial memory. However, the lack of improvement observed in the same group once the drugs were removed suggests that DCS should be administered daily. Future research on DCS should focus on proper dose and duration of treatment in order to optimize the benefits of DCS. Tsai et al. (1999) found that DCS significantly improved cognition in human patients with AD; however, the study was acute with patients only receiving DCS for a

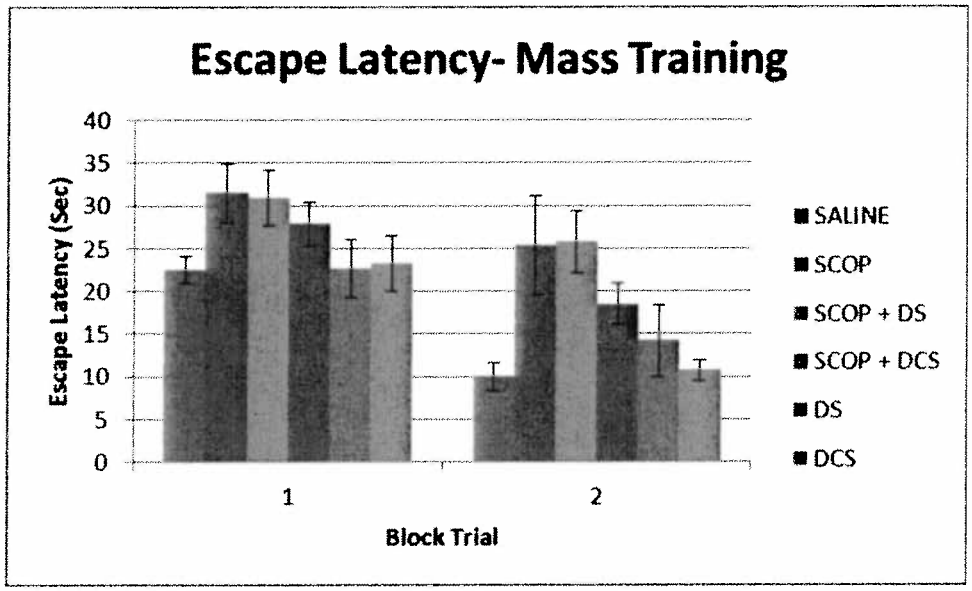
few weeks. It seems that given the positive results of this and other investigations, DCS should be retested in the human population for a longer duration of time.

Figure 1. Escape Latency to Hidden Platform During Phase 1 of CPT



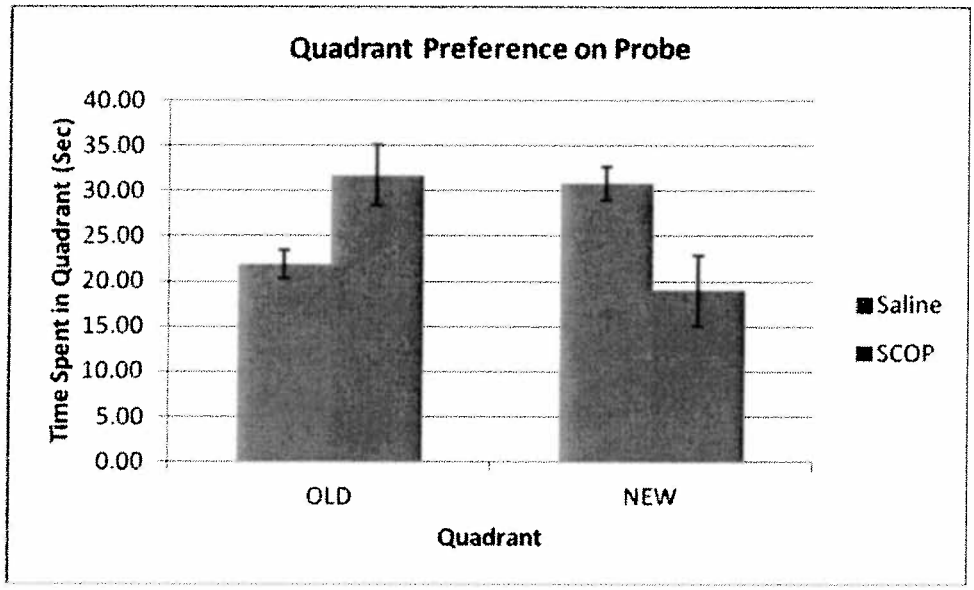
There was no significant difference found between groups during Phase 1 of the CPT. Animals in all groups performed at a statistically equal pace indicating equality in learning potential prior to drug influence.

Figure 2. Escape Latency during Phase 2 of CPT



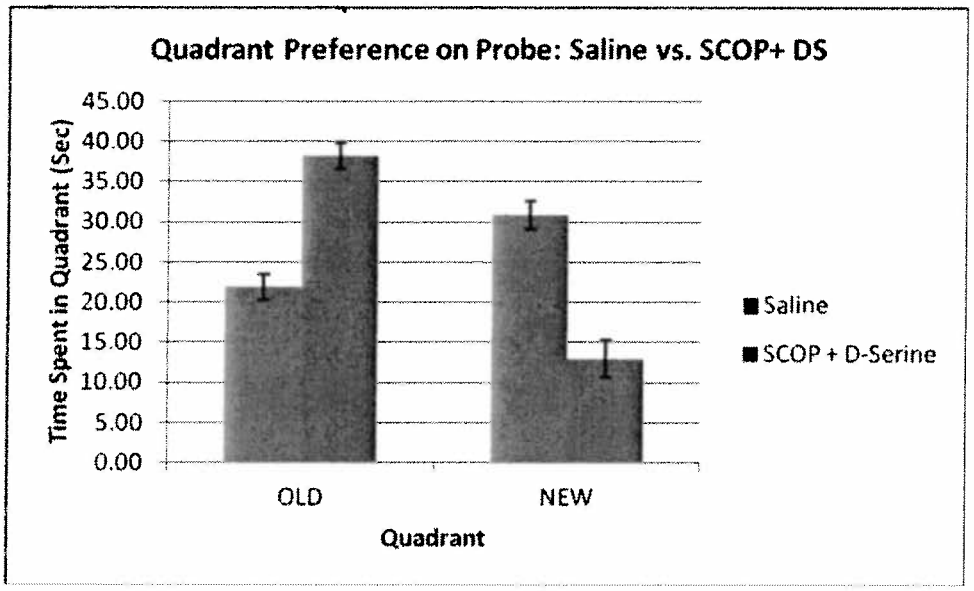
A 6 x 2 (Group x Block) mixed ANOVA (Group: Saline, SCOP, SCOP + DS, SCOP +DCS, DS, DCS; Block: average escape latencyTrials 1-4, average escape latency Trials 5-8) revealed a significant main effect of Group. Further analysis indicated the the Saline group performed significantly better than the SCOP and SCOP + DS groups. Results indicated that the SCOP + DCS, DS alone and DCS alone were not significanly different than that of the Saline group. Additionally, escape latency for the DCS alone group was significantly faster than those of animals treated with SCOP and animals treated with SCOP + DS. No other significant differences were found between grous;

Figure 3. Amount of time spent in Old vs. New quadrant during probe test



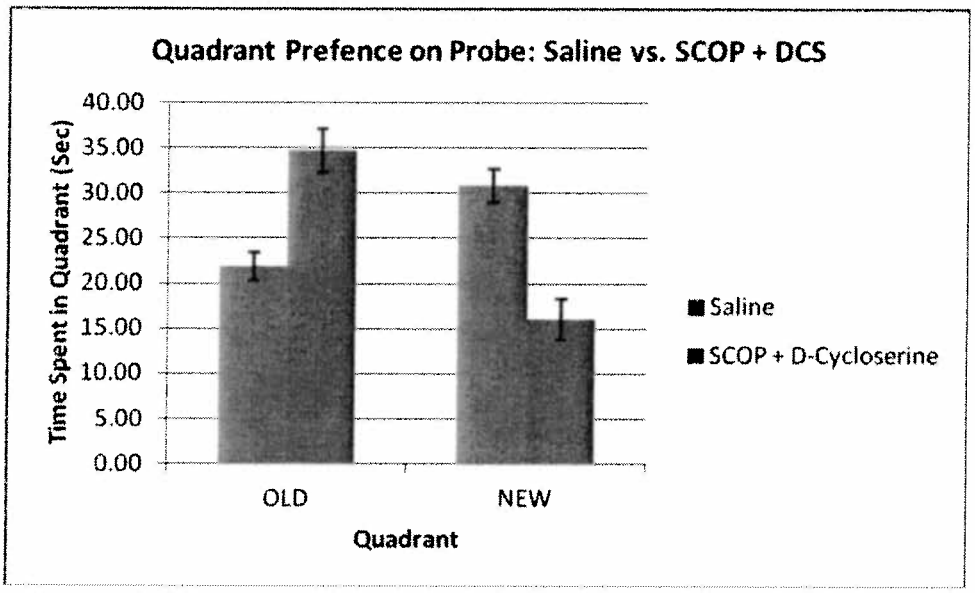
A 2 x 2 (Group x Quadrant) mixed ANOVA with repeated measures on the second factor (Group: Saline and SCOP; Quadrant: Old and New) revealed a significant cross over interaction between group and quadrant. These results suggest that compared to control, SCOP-treated animals spent significantly more time searching the Old location quadrant.

Figure 4. Amount of time spent in Old vs. New quadrant during probe test. Saline vs. SCOP + DS Groups



A 2 x 2 (Group x Quadrant) mixed ANOVA with repeated measures on the second factor in which group included Saline and SCOP + DS and Quadrant was Old and New location resulted in a significant main effect of quadrant $F(1, 44)=18.63$, $P<.001$ and a significant interaction between group and quadrant $F(1, 44)=82.169$, $P<.001$. These results indicate that the SCOP + DS-treated rats demonstrated a significant preference for the old location compared to the new quadrant location. This preference for the old quadrant location was also significantly greater than that of the saline-treated rats which spent significantly more time in the new location quadrant.

Figure 5. Amount of time spent in Old vs. New quadrant during probe test. Saline vs. SCOP + DCS Groups



A 2 X 2 (Group x Quadrant) mixed ANOVA with repeated measures on the second factors (group: Saline and SCOP + DCS; Quadrant: New and Old) revealed a main effect of quadrant $F(1, 44)=5.194$, $P<.05$ and a significant interaction of group and quadrant $F(1, 44)=41.972$, $P<.001$. These results indicate that compared to control, rats treated with SCOP and DCS spent significantly more time searching the area of the old location on the day of the probe test. The preference for the old quadrant location was also significantly greater than the preference shown by the same group for the new location. Rats in the saline (Control) condition behaved in an opposite fashion, spending a significantly greater amount of time swimming in the new quadrant location compared to the old location.

Figure 6. Amount of time spent in Old vs. New quadrant during probe test. Saline vs. DCS alone group.

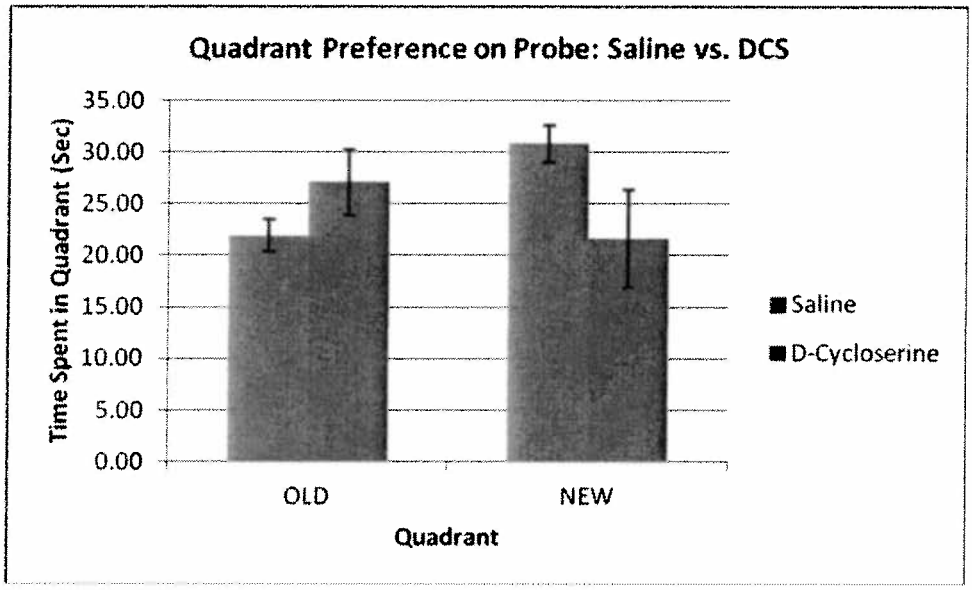


Figure 6 represents the results of a 2 X 2 (Group x Quadrant) mixed ANOVA with repeated measures on the second factors (group: Saline and DCS; Quadrant: New and Old) and revealed significant interaction between group and quadrant $F(1, 34)=6.709$, $p<.05$. These results suggest that rats treated with DCS demonstrated an overall preference for the old quadrant location compared to control. Control rats treated with Saline, showed a showed partiality for the new quadrant location. There was no significant preference for either the old or the new quadrant location when the DCS group was compared alone.

Figure 7. Amount of time spent in Old vs. New quadrant during probe test. Saline vs. DS groups.

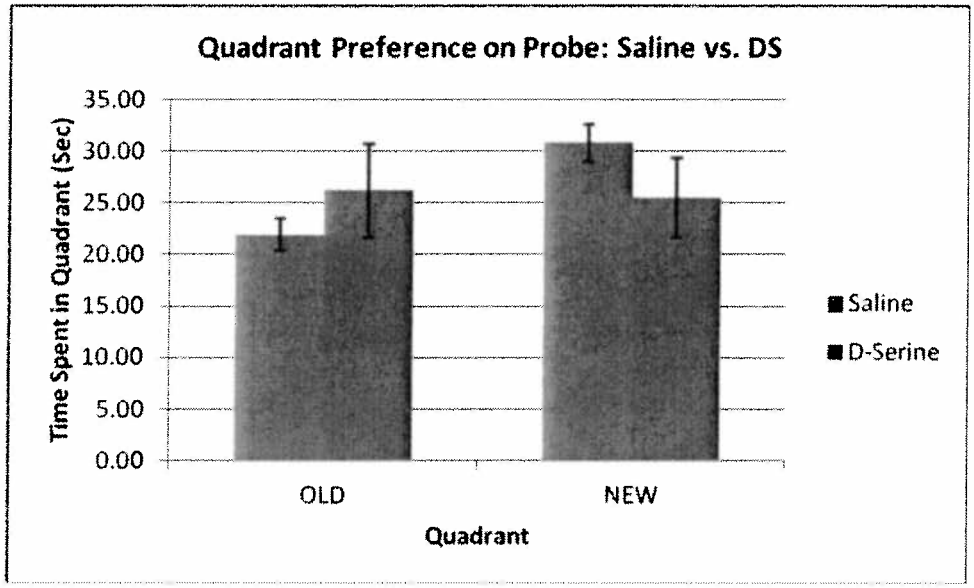


Figure 7 represents the results of a 2 x 2 (Group x Quadrant) mixed ANOVA with repeated measures on the second factor (Group: Saline and DS; Quadrant: Old and New). These results of this analysis suggest that there were no significant differences between the observed behavior of the Saline and DS alone groups. The DS alone gorup did not demonstrate a significant preference for either the old or the new quadrant location, spending equal amounts of time searching both.

Figure 8. Amount of time spent in Old vs. New quadrant during probe test, a comparison of all groups.

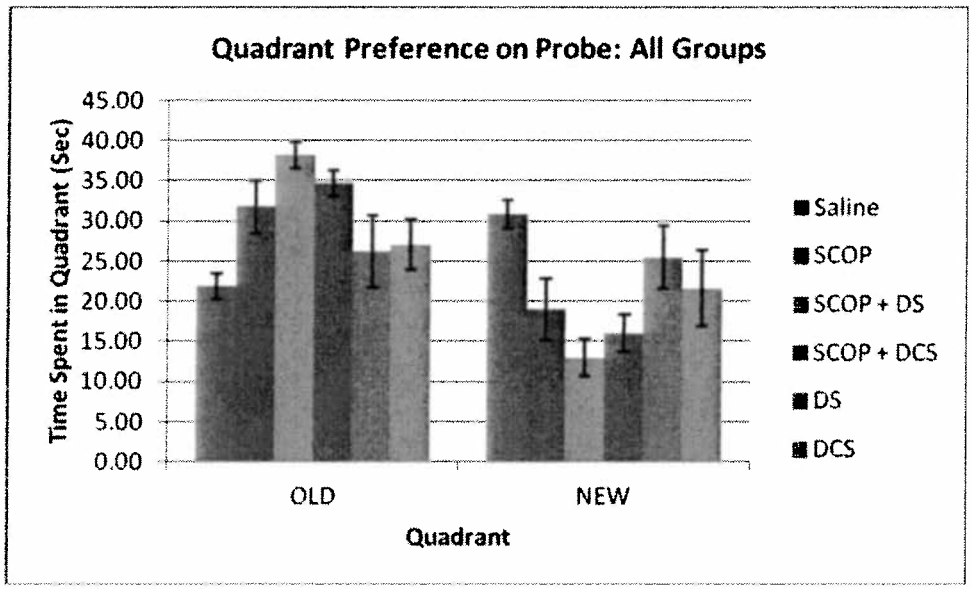


Figure 8 represents a compilation of all group results during the probe trail (Phase 3).

APPENDIX A

Hi Charlotte,

Here is the IACUC approval email for the protocol that includes your MA thesis. This should be included in your thesis documents.

Sincerely,

Dr. Devan

From: Liliana Rao [mailto:LRao@vetmed.umaryland.edu]
Sent: Friday, June 03, 2011 2:19 PM
To: Devan, Bryan
Subject: Re: Revised Protocol (formerly #F0405RPR.02)

Dear Dr Devan,

I am writing to inform you of the approval of the protocol **IACUC # 060311 BD 01** in title **" Cognitive enhancement of learning and memory in rats "**

The approval for this protocol is, June 3, 2011. Your protocol is approved for a period of 3 years.

(Please update the literature research and submit the new one to me as soon as possible along with the room numbers that you will use for working with the animals.

Dr Louis DeTolla, Chair, IACUC TU will send an official letter of approval in near future.

Best Regards,

Liliana Rao

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CURRICULUM VITA

NAME: Charlotte Eyring

PERMANENT ADDRESS: 1216 Cooksie st.
Baltimore, Maryland, 21230

PROGRAM OF STUDY: Experimental Psychology

DEGREE TO BE OBTAINED: Master of Arts., 2012

Secondary Eduaction: Towson University
Baltimore, Maryland
2012

Collegiate institutions attended	Dates	Degree	Date of Degree
Towson University	2009-2012	Master of Art	2012
Gettysuburg College	2001-2005	Bachelor of Art	2005

Major: Psychology

Minor: Religion

Professional Publications:

Blue, M., Kaufmann, W., Bressler, J., Eyring, C., Odriscoll, C., Naidu, S., Johnston, M. (2011).Temporal and regional alterations in NMDA receptor expression in *Mecp2*-Null mice. *The Anatomical Record*, 294 (10): 1624-1634.

Professional Positions Held:

Research Assistant: Neuroscience Lab at Kennedy Krieger Institute
707 N. Broadway St.
Baltimore, Maryland, 21205
President: The Baker- King Fund Incorporated
Baltimore, Maryland