Spring 2009

Effect of cholinergic deafferentation of prefrontal cortex on working memory for familiar and novel odors

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EFFECT OF CHOLINERGIC DEAFFERENTATION OF PREFRONTAL CORTEX ON WORKING MEMORY FOR FAMILIAR AND NOVEL ODORS

BY

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B.A., University of New Hampshire, 2007

THESIS

Submitted to the University of New Hampshire

in Partial Fulfillment of

the Requirements for the Degree of

Master of Arts

in

Psychology

May, 2009

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4/17/09
Date

ACKNOWLEDMENTS

I would like to express my appreciation for my advisor, Dr. Jill McGaughy, for her guidance over the past two years and for her inspiring passion for research. I would also like to thank the members of my committee, Dr. Rob Drugan and Dr. Andy Leber, for their interest and suggestions concerning this project, as well as Lori Newman for her mentorship in the lab.

Finally, I want to thank my family for their unconditional love and support: my parents for always believing in me, my sister Nicole for being my other half, and my fiancé Bryan for his unwavering encouragement and optimism. I couldn't have done it without you.

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ABSTRACT

EFFECT OF CHOLINERGIC DEAFFERENTATION OF PREFRONTAL CORTEX ON WORKING MEMORY FOR FAMILIAR AND NOVEL ODORS

by

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The role of acetylcholine in the medial prefrontal cortex (mPFC) in working memory was investigated in aged rats. Subjects with cholinergic lesions of the prelimbic portion of the mPFC (pACh-lx) or a sham lesion of the same region were trained on an odor delayed non-match to sample paradigm. The effects of prefrontal cholinergic depletion and aging were assessed in task variations that manipulated mnemonic demand and stimulus novelty.

pACh-lx animals were impaired relative to sham-lx animals at memory for familiar stimuli over delays. This global impairment was not dependent on the length of the delay, suggesting that aged pACh-lx animals were impaired at a non-mnemonic component of the working memory task (e.g., see page 7). Aged animals' accuracy was also impaired during sessions with novel sample stimuli, indicating a novel encoding impairment due to age-related cholinergic depletion in the entorhinal cortex.

CHAPTER I

INTRODUCTION

Executive functioning refers to the cognitive processes necessary to perform widespread cognitive tasks including learning, planning, decision-making, task-switching, and problem-solving (Baddeley & Della Sala, 1996; Goldman-Rakic, 1990, 1996; Robbins, Weinberger, Taylor, & Morris, 1996; Dalley, Cardinal, & Robbins, 2004). Such tasks require the ability to attend to incoming sensory information as well as the ability to hold, manipulate, and update this information as necessary (Baddeley & Hitch, 1974; Baddeley & Della Sala, 1996; Goldman-Rakic, 1996; Robbins, et al., 1996, Müller & Knight, 2006). This ability to maintain and organize information as it is held "online" is referred to as working memory (Baddeley & Hitch, 1974; Goldman-Rakic, 1990).

Working memory is hypothesized to be a limited capacity system (Baddeley & Hitch, 1974; Baddeley & Della Sala, 1996). Performance decrements result, either in terms of accuracy or response latency, when memory load increases past available processing resources (Baddeley & Hitch, 1974; Baddeley & Della Sala, 1996). Mnemonic processing can be influenced by the period of time a stimulus must be remembered (Goldman-Rakic, 1990, 1996; Dunnett, 1990, 1992; Funahashi, Bruce, & Goldman-Rakic, 1993; Sloan, Good, & Dunnet, 2006), by the number of stimuli to be remembered (Petrides, 1995,

2000; Eacott, Gaffan, & Murray, 1994; Newman & McGaughy, 2009), or by external factors such as aging, which is associated with a reduction in processing resources (Dunnett, 1992; Aggleton, Blindt, & Candy, 1989; Mattay et al., 2006).

Working memory can be assessed in humans and animals with a variety of cognitive tasks that require the maintenance, manipulation, or monitoring of sensory stimuli (Baddeley & Hitch, 1974; Goldman-Rakic, 1996; Robbins, Weinberger, Taylor, & Morris, 1996). Delayed-response tasks require a subject to remember a stimulus over a delay period, then make a response that indicates memory for the initial stimulus (Goldman-Rakic, 1990, 1996; Funahashi et al., 1993). Variations of this task include delayed match or non-match to position (DMTP/DNMTP), which assesses spatial working memory by requiring an animal to remember which arm of a T-maze was previously entered or which of two levers was pressed (Granon, Vidal, Thinus-Blanc, Changeux, & Poucet, 1994; Dunnett, 1990, 1992; Sloan et al., 2006). Delayed match or non-match to sample (DMS/DNMS) requires memory for the features of an object stimulus over a delay (Gaffan & Murray, 1992; Eacott, Gaffan, & Murray, 1994; Suzuki, Miller, & Desimone, 1997; McGaughy, Koene, Eichenbaum, & Hasselmo, 2004, 2005; Schon, Hasselmo, LoPresti, Tricarico, & Stern, 2004; Schon, Atri, Hasselmo, Tricarico, LoPresti, & Stern, 2005; Turchi, Saunders, & Mishkin, 2005). Similarly, the N-back task requires a subject to recognize from a sequential presentation of stimuli the one that they saw N trials back (Mattay et al., 2006; Stern, Sherman, Kirchhoff, & Hasselmo, 2001; Tsuchida & Fellows, 2008).

Performance at such working memory tasks has been shown to be modulated by the neurotransmitter acetylcholine (ACh) in the prefrontal cortex (PFC; McGaughy et al., 2004; Chudasma, Dalley, Nathwani, Bouger, & Robbins, 2004; Schon et al., 2005), particularly when memory for familiar information is required (Schon et al., 2005; Stern et al., 2001). In addition, by varying the familiarity of the stimulus to be remembered, delayed-response tasks can assess other areas of the brain known to mediate memory for novel information, particularly the entorhinal cortex (EC) within the medial temporal lobe (McGaughy et al., 2005; Stern et al., 2001; Stern et al., 1996).

The present study examined the effects of cholinergic lesions of the prelimbic cortex, a subregion of the medial prefrontal cortex, on aged rats performing an odor delayed non-match to sample task. As cholinergic activity in this region is thought to be necessary for the processes involved in working memory (Carballo-Marquez, Vale-Martínez, Guillazo-Blanch, Torras-Garcia, Boix-Trelis, & Martí-Nicolovius, 2007; Power, Vazdarjanova, & McGaugh, 2003; Hasselmo & McGaughy, 2004) it was predicted that lesioned animals would be impaired at the prefrontally-mediated components of the DNMS task, namely, memory over delays and memory for lists of multiple stimuli. However, aging itself has been associated with deficits on prefrontally-mediated tasks (Rapp & Amaral, 1989; Dunnett, 1992; Aggleton, Blindt, & Candy, 1989) that are possibly due to age-related impairments in attentional and encoding abilities that affect working memory performance (Jones, Barnes, Kirkby, & Higgins, 1995; McGaughy & Sarter, 1995; Moore, Dudchenko, Bruno, & Sarter, 1992). It was

therefore predicted that in addition to prefrontally-mediated mnemonic impairments, it was possible that animals in the current study would show age-related impairments related to the non-mnemonic processes vital to the performance of working memory tasks. Finally, based on the role of the entorhinal cortex in novel encoding (McGaughy et al., 2005) and the deterioration of this region with aging (Gomez-Isla et al., 1996; Perry, 1998), it was also hypothesized that age-related cholinergic depletion in this area would produce impairments on the DNMS task preferentially for novel information, distinct from the effects of the pACh-lesion.

CHAPTER II

THE PREFRONTAL CORTEX AND MEMORY

The prefrontal cortex is hypothesized to be critical to executive functioning (Goldman-Rakic, 1990; Baddeley and Della Sala, 1996), and widespread memory and attentional impairments result when this area is disrupted pharmacologically or by lesioning (Otto & Eichenbaum, 1992a; Baddeley & Della Sala, 1996; Goldman-Rakic, 1996; Petrides, 2000; Chudasama, Dalley, Nathwani, Bouger, & Robbins, 2004; Dalley, Theobald, Bouger, Chudasama et al., 2004; McGaughy et al., 2004; Newman & McGaughy, 2008). The dorsolateral prefrontal cortex in humans and monkeys and its anatomical equivalent in rats, the medial prefrontal cortex (Vertes, 2004) is thought to specifically play a role in working memory by maintaining a representation of a stimulus in its absence (Goldman-Rakic, 1990; 1996; Funahashi et al., 1993).

Single-cell recordings in monkeys have demonstrated that the PFC contains subsets of neurons that respond to the sensory input of information, to maintaining a representation of that stimulus, and to the behavioral output or motor response (Goldman-Rakic, 1996). Of particular importance is the subset of neurons in the PFC that fire to maintain a representation of a stimulus once it has been removed (Goldman-Rakic, 1996). These PFC neurons appear to be highly specialized in that they encode stimulus information from a distinct location; if

these neurons are inactivated, memory for stimuli in that particular location is impaired while memory for other locations is spared (Goldman-Rakic, 1996).

Damage to the PFC has been shown to cause impairments in a range of working memory tasks in monkeys, humans and rats (Petrides, 1995, 2000; Funahashi, Bruce, & Goldman-Rakic, 1993; Petrides & Milner, 1982; Dunnett, Wareham, & Torres, 1990; Granon et al., 1994; Sloan et al., 2006). For example, monkeys with ablations of the dorsolateral prefrontal cortex were impaired at a delayed response task where they were required to fixate on the location of a previous cue after a delay period (Funahashi et al., 1993). Monkeys with ablations of the mid-dorsolateral prefrontal cortex (dlPFC) performed at chance levels on a self-ordered working memory task where they were required to choose a different object on each of three trials to receive a reward (Petrides, 1995). They were also impaired on an externally-ordered working memory task where they were presented with the first two stimuli and had to choose the one object of the three they had not yet seen (Petrides, 1995). Rats with PFC lesions were impaired at delayed non-match to position tasks both in a T-maze (Granon et al., 1994) and with lever stimuli (Dunnet, Wareham, & Torres, 1990; Sloan et al., 2006). Human subjects with frontal lobe lesions were impaired relative to normal controls at a self-ordered task similar to the one used in monkeys, where they were presented with sets of pictures on sheets of paper and required to touch each image only once without repeating (Petrides & Milner, 1982). At each type of task, the PFC is necessary for maintaining a representation of the stimulus in order to respond correctly.

Damage to the PFC also has been shown to cause impairments in memory when a delay is imposed between the onset of the stimulus and the required response (Goldman-Rakic 1990; 1996; Petrides, 1995; Eacott, Gaffan, & Murray, 1994). However, there are contrasting reports as to whether such deficits are dependent on the length of the delay period, or whether the addition of any delay impairs performance. When memory for stimuli deteriorates as the delay period increases, this has been taken as evidence of a mnemonic deficit, supporting the hypothesis that relevant information can only be held online for a limited period of time (Dunnett, 1990; Sloan et al., 2006). Some research has supported a role for the PFC in such mnemonic processing (Dunnett, 1990; Sloan et al., 2006; Rapp & Amaral, 1989; Funahashi et al., 1993). For example, Dunnett (1990) found that rats with discrete excitotoxic lesions to the mPFC were increasingly impaired on a DNMTP task as the delay increased from zero to 24 seconds. Other researchers have found that memory for stimuli is impaired at all delays (i.e., Chudasama et al., 2004), suggesting that a non-mnemonic process is compromised, such as the ability to attend to or encode the relevant information (Dunnett, 1990; Petrides, 2000; Müller & Knight, 2006). Previous studies tested delays ranging from two to 30 seconds (Dunnett, 1990; Rapp & Amaral, 1989; Funahashi et al., 1993; Sloan et al., 2006). The current study aimed to maximize mnemonic demand by requiring subjects to remember stimuli over delays on the order of several minutes to an hour.

The neurotransmitter acetylcholine (ACh) has been implicated in mediating many cognitive processes (Carballo-Marquez, Vale-Martínez, Guillazo-

Blanch, Torras-Garcia, Boix-Trelis, & Martí-Nicolovius, 2007; Power, Vazdarjanova, & McGaugh, 2003; Hasselmo & McGaughy, 2004). The activity of acetylcholine has been shown to enhance encoding of stimuli, consolidate newly learned information, reduce interference of distracting information, and to direct attention to task-relevant behavior (Herremans, Hijzen, Welborn, Olivier, & Slangen, 1996; Hasselmo & McGaughy, 2004; Carballo-Marquez et al., 2007).

A role for ACh in the PFC in working memory has been well-established, particularly in memory for familiar stimuli (Dunnett, 1990; McGaughy, Koene, Eichenbaum, & Hasselmo, 2004; McGaughy, Koene, Eichenbaum, & Hasselmo, 2005). Computational models suggest that acetylcholine neurons in the PFC exhibit sustained spiking activity in response to familiar stimuli (Durstewitz, Seamans, & Sejnowki, 2000; McGaughy et al., 2005). This sustained spiking is thought to maintain the representation of a familiar stimulus in its absence, supported by the fact that cholinergic lesions of the mPFC in rats impair odor delayed non-match to sample performance for familiar stimuli (McGaughy et al., 2004; Newman & McGaughy, 2009). These animals are particularly impaired at the DNMS task when they explore the match (incorrect) cup first. Subjects with PFC lesions are thought to rely on other brain regions including the EC for encoding the familiar sample; however, neurons in these regions do not fire continuously in response to familiar stimuli. It has been hypothesized that when these animals encounter a repetition of the sample odor, i.e., the incorrect response, the EC begins a new firing pattern and the representation of the original sample is lost (McGaughy et al., 2004).

To make a correct choice at the standard DNMS task, a subject must be able to remember the sample they encountered immediately preceding the current trial. During this type of testing, odors prior to the preceding odor are no longer relevant and it is beneficial not to maintain them. When stimuli are highly familiar or repetitive, this increases the risk that memory for stimuli seen previously will interfere with memory for the current trial. For example, in delayed non-match to position tasks where the sample stimuli is one of two levers (i.e., Dunnett, 1990, 1992; Sloan et al., 1996) and delayed non-match to sample tasks that use only two objects (i.e, Petrides, 1995; Eacott, Gaffan, & Murray, 1994), the subject must not only remember the current sample but must also monitor the temporal order of the preceding trials to avoid interference effects (Petrides, 1995). The activity of ACh in the prefrontal cortex in response to familiar stimuli is thought to help avoid this proactive interference by monitoring the information and keeping it organized (Otto & Eichenbaum, 1992a; Petrides, 1995, 2000; Stern et al., 2001; McGaughy et al., 2004).

The risk of proactive interference is also increased when multiple stimuli must be maintained in memory at once, particularly when the information to be remembered is highly familiar. In Petrides' (1995) study on self-ordered working memory, he varied the size of the initial set of familiar stimuli to be monitored, and found that the monkeys with ablations of the mid dorsolateral prefrontal cortex were highly impaired when required to monitor three familiar objects instead of two. In rats, the list variation of the DNMS task assesses memory for multiple stimuli, increasing the risk of proactive interference from previous trials

during sequential choice phases (Newman & McGaughy, 2009). Such studies provide evidence that ACh specifically mediates this monitoring of familiar information, as cholinergic lesions of the prelimbic portion of the mPFC cause deficits in memory for lists of several familiar odor stimuli (Newman & McGaughy, 2009).

Based on the role of acetylcholine in the prefrontal cortex in organizing familiar information, it was hypothesized that in the current study pACh-lx animals would be impaired at memory for multiple stimuli and that this impairment would increase at longer list lengths. Additionally, due to the role of ACh in the PFC in memory over delays, it was predicted that pACh-lx animals would be increasingly impaired at longer delay periods. Specifically, it was predicted that sham-lx and pACh-lx would perform similarly at short delays; as delay was increased, sham-lx animals would become gradually impaired while pACh-lx performance would deteriorate in a more pronounced manner.

CHAPTER III

AGING AND MEMORY

Aging is associated with loss of cholinergic neurons throughout the entire cortex (Baxter, Frick, Price, Breckler, Markowska, & Gorman, 1999; Perry et al., 1998; Geula & Mesulam, 1996). Aging has been shown to impair attention (McGaughy & Sarter, 1995; Moore et al., 1992; Jones et al., 1995), as well as impair acquisition and performance of working memory tasks including delayed-match to sample (Aggleton et al., 1989; Dunnett, Martel, & Iverson, 1990). Working memory impairments in aged rats have been shown to correlate with the amount of ACh fiber loss in several brain regions including the frontal cortex and entorhinal cortex as measured by levels of choline acetyltransferase (ChAT; Baxter et al., 1999).

Behavioral tasks mediated by the prefrontal cortex have been shown to be impaired with aging (Rapp & Amaral, 1989; Dunnett, 1992; Mattay et al., 2006). Rapp and Amaral (1989) found that aged monkeys were impaired at delayed non-match to sample with two familiar objects. The aged monkeys were impaired at monitoring for proactive interference, which was increased in this task by requiring the animal to remember the temporal order of preceding trials in order to make a correct response (Rapp & Amaral, 1989).

In humans, aging may impair the functioning of the prefrontal cortex leading to impairments in working memory, particularly when cognitive demands are increased (Mattay et al., 2006). Older subjects performed just as accurately as younger controls at a 1-back task of working memory using a small set of highly familiar stimuli, but their reaction times were slower and fMRI analysis showed greater prefrontal activation during task performance, suggesting that they were compensating for reduced processing resources (Mattay et al., 2006). Older subjects' performance was impaired at 2- and 3-back tasks using the same stimuli, versions of the task that increase proactive interference. This supports the idea that the aged individuals' processing limits were reached at lower levels of task complexity compared with younger subjects performing the same tasks (Mattay et al., 2006).

Aged related cholinergic fiber loss in the brain occurs in a caudal to rostral pattern (Geula & Mesulam, 1996; Perry et al., 1998). Therefore, one of the brain areas where cholinergic fiber loss occurs in the earliest stages of aging is the entorhinal cortex (EC; Hyman et al., 1984; Gomez-Isla et al., 1996). This region has been shown to mediate memory for novel information (Stern et al., 1996; Schon et al., 2004; McGaughy et al., 2005). Acetylcholine neurons in the entorhinal cortex show persistent spiking in response to novel stimuli after the actual stimulus is no longer present, holding a representation available until it can be encoded into long term memory by connections with the hippocampus and prefrontal cortex (Klink & Alonso, 1997; Koene et. al, 2003; McGaughy et al., 2005).

Damage to this area causes extensive impairments in memory for trial-unique stimuli (Zola-Morgan et al., 1989; Gaffan & Murray, 1992; Eacott, Gaffan, & Murray, 1994; Tang, Mishkin, & Aigner, 1997; Turchi et al., 2005) while leaving memory for familiar stimuli largely intact (McGaughy et al., 2007; McGaughy et al., 2005). In monkeys, rhinal cortex ablations impaired DNMS performance for trial-unique objects, but these animals were indistinguishable from controls at DNMS task using two highly familiar objects (Eacott, Gaffan, & Murray, 1994). Turchi et al. (2005) demonstrated that cholinergic lesions of the rhinal cortex in monkeys were sufficient to produce the same effects at the DNMS task as ablation lesions, indicating the role of the cholinergic system in this region in memory for novel information. McGaughy et al. (2005) directly tested the role of acetylcholine in the entorhinal cortex in memory for novel information. These results demonstrated that cholinergic lesions of the EC in rats impaired DNMS performance for novel odors but not for odors that were highly familiar (McGaughy et al., 2005). Studies in humans also provide evidence that the cholinergic system in the rhinal cortex is critical to memory for novel information. When acetylcholine was blocked pharmacologically in humans by the administration of scopolamine, a muscarinic antagonist, subjects were impaired at a delayed match to sample task using novel pictures; fMRI scanning demonstrated that this impairment was correlated with reduced activity in the parahippocampal regions including the entorhinal cortex (Schon et al., 2005).

The role of acetylcholine in the entorhinal cortex in encoding novel information is particularly important when considering that early memory

impairments seen in humans with Mild Cognitive Impairment and Alzheimer's disease are thought to result from the loss of cholinergic neurons in this region (Hyman et al., 1984, Gomez-Isla et al., 1996; Whitwell et al., 2007). Gomez-Isla et al (1996) found that even people with a diagnosis of Mild Cognitive Impairment had significantly fewer neurons in the EC than controls, and Whitwell et al. (2007) found that minor atrophy in the medial temporal lobes was predictive of an Alzheimer's Disease diagnosis three years later.

The subjects in the current study were approximately 18 months old. While some studies have used rats of this age as models of cognitive aging (i.e., Moore et al., 1992), other studies have used subjects between 24-28 months old (Wilson et al., 2004; Sugaya et al., 1996), so subjects in the current study were in the middle stages of aging. It was predicted that because the entorhinal cortex is located in the caudal region of the brain where deterioration starts earlier, the animals in the current study were old enough to have cholinergic depletion in this area. It was therefore hypothesized that aged animals, regardless of lesion, would be impaired on the delayed non-match to sample task when novel stimuli were used.

CHAPTER IV

CURRENT EXPERIMENT

The current experiment was designed to test the two hypotheses discussed above:

1. Animals with cholinergic lesions of the prelimbic cortex would be impaired at prefrontally-mediated tasks, including memory for stimuli over delay periods and memory for lists of multiple stimuli.

2. All aged animals would have impairments in encoding novel information due to age-related cholinergic fiber loss in the entorhinal cortex, relative to their own performance at sessions with familiar odors.

To address these hypotheses, aged animals with preexisting cholinergic lesions of the prelimbic cortex (pACh-lx) or sham lesions of the same area were trained on a delayed non-match to sample with odors. Animals acquired the task using the same set of highly familiar stimuli. After reaching high levels of accuracy, variations were added to the sessions with familiar stimuli increasing the cognitive demand. Memory for novel odors was then assessed and compared with performance for familiar odors to examine age-related decreases in this form of memory.

CHAPTER V

METHOD

Subjects

14 male, Long-Evans hooded rats were used in this study. They were moderately food-restricted to allow each rat to maintain a weight that was approximately ~90% of free feed weight of age-matched controls with water available *ad libitum*. They were housed individually on a 12L/12D cycle with lights on at 6 am. At approximately 16 months of age (400-500 g), animals began training in the Delayed Non-Match to Sample (DNMS) task. DNMS training took place between 8:00 am and 12:00 pm, 6 days a week. All procedures were reviewed and approved by IACUC at the University of New Hampshire.

Surgical Procedures

Animals received surgery between 8 and 10 months of age as part of an earlier study of crossmodal divided attention. Seven subjects received a cholinergic lesion of the medial prefrontal cortex centered on prelimbic cortex (pACh-lx) while 7 received a sham lesion of the same area (sham-lx).

Animals were anesthetized with ketamine (85 mg/kg) and xylazine (8.5 mg/kg) and placed in a stereotaxic frame using blunt ear bars and a tooth bar.

Petroleum jelly was placed on the eyes to keep them from drying. Animals assigned to receive the pACh-lesion received infusions of the toxin 192-IgG-saporin (0.10 µg/ µl in Dulbecco's saline) and animals assigned to receive sham lesions received infusions of vehicle (Dulbecco's saline) into the prelimbic cortex at coordinates of: AP: +2.8, ML: ±0.6, DV: -5.2 (anteroposterior measurement relative to Bregma, dorsoventral measurement relative to the skull and toothbar at -3.3). Infusions were made by a 10 µl, 26 gauge microsyringe via an infusion pump (Micro 4™: Microsyringe Pump Controller; World Precision Instruments, Sarasota, FL) bilaterally at 125 nl per second. The needle was left in place for four minutes prior to and following the infusion. Following surgery, animals were given *ad libitum* food and water for seven days before food restriction was reinstated. Animals resumed behavioral training at the crossmodal divided attention task 10 days after surgery. An average of 4.6 $\pm$ 0.3 months (MEAN $\pm$ SEM) passed between surgery and the onset of DNMS training (range: 2.5 – 6.9 months).

DNMS training

Apparatus

Behavioral training took place on a 91.3 X 91.3 cm black Plexiglas board with 24 pieces of Velcro® (Velcro USA Inc., Manchester, NH) affixed equidistantly around the perimeter of the board and one piece affixed to the center of the board. Plastic cups (4 oz, 118 ml, 7 cm diameter x 5 cm high,

Glad®, Cartersville, GA) filled with sand (Worldwide Imports, Enterprise, Inc. Fort Lauderdale, FL) and scented with common household spices (i.e., ginger, thyme, mint) were used as stimuli. Each cup had a strip of Velcro® attached to its underside to be placed on the Velcro® strips on the board to prevent tipping and spilling.

Shaping

Animals were first placed on the Plexiglas board with food reinforcement (45 mg Pellets, Bio Serv Frenchtown, NJ) sprinkled around the surface of the board. A plastic cup of scented sand with food pellets on top placed in the center of the board. Animals were given ~15 minutes to explore the board and retrieve the reinforcement from the board and the cup. The following day animals were presented with only the cup of scented sand with food pellets on the top, and were allowed to retrieve the reinforcement. After the rat retrieved the reinforcement, he was removed from the board and the board was cleaned with 70% ethanol to remove traces of previous odors. A cup with a new odor was presented and the rat was again allowed to retrieve the reinforcement. This process was repeated for 20 trials per day, using the 20 odors that the rats would be trained with in the DNMS task. These odors to which the animals were repeatedly exposed were considered the familiar odors. Rats received 34 $\pm$ 8.2 (Mean $\pm$ SEM) days of exposure to these scents from the beginning of shaping to the onset of testing with a new set of odors.

When animals could reliably retrieve the reinforcement from the top of the cup, the food pellets were buried progressively deeper in the sand on each trial until the animal had to dislodge sand in order to retrieve reinforcement. Animals were given a limited hold of 90 seconds to dig in the sand and retrieve the food pellet; otherwise, they were removed from the board and the next trial was presented. Animals took an average of 2-3 days until they could reliably dig for a fully buried food pellet, after which they began training on the DNMS task.

DNMS task

A cup with a sample odor was placed in the center of the board. The animal was given a limited hold of 90 seconds to dig in the cup and retrieve the food reward (45 mg Pellets, Bio Serv Frenchtown, NJ). After he retrieved the reinforcement, the rat was removed from the board which was wiped clean with 70% ethanol. Two choice cups were then placed on the perimeter of the board, separated by 24 centimeters. The match cup was a clone of the sample cup and the non-match cup was scented with a different odor from the set of familiar odors. A clone of the sample cup rather than the sample cup itself was used so that the animal could not mark the cup during the sample phase in a way that would facilitate correct responding. Animals were given 90 seconds to make a response, and were reinforced for digging in the non-match cup. If they dug in the match (incorrect) cup, they were removed from the board without reinforcement and the next sample cup was presented. If the animal failed to respond after 90 seconds the trial was considered an omission, and they were

removed from the board without reinforcement and the next trial was presented. This process was repeated 10 times in one session. The same 20 odors were used during training so that they became highly familiar. Trials were counterbalanced so that the sample/correct nonmatch pairings varied each day and each odor appeared sometimes as the sample and sometimes as the correct nonmatch. The placement of the two choice cups was also varied between the four sides of the board and the correct non-match was randomly varied between the right and left side, to discourage the use of spatial cues. During this phase of testing, the delay period between the sample and choice phases was equivalent to the time taken to clean the board, approximately 20 seconds.

Criterion performance was defined as $\geq$ 8/10 correct over two consecutive sessions. When animals reached criterion performance, the two choice cups were moved to 48 cm apart, and when they could reliably perform at this level, the choice cups were moved to the corners of the board, 72 cm apart (See Figure 1). Familiar odor training was arranged so that half the rats were exposed to one set of 20 familiar odors and the other half were exposed to a different set; the particular odor set that each rat was not exposed to during familiar training later became the novel odor set for that subject.

Tests of differential mnemonic demand

After animals reached criterion performance at the standard DNMS task when choice cups were 72 cm apart, they were presented with task variations

Figure 1. *Birds-eye view of testing apparatus. Left: the sample cup (i.e. tea), is presented in the center of the board and the animal is allowed 90 s to retrieve the reward. Right: the choice phase; the animal is placed in the center of the board equidistant from the two choice cups and is given 90 s to respond. The rat is reinforced for choosing the non-match cup (i.e., clove).*

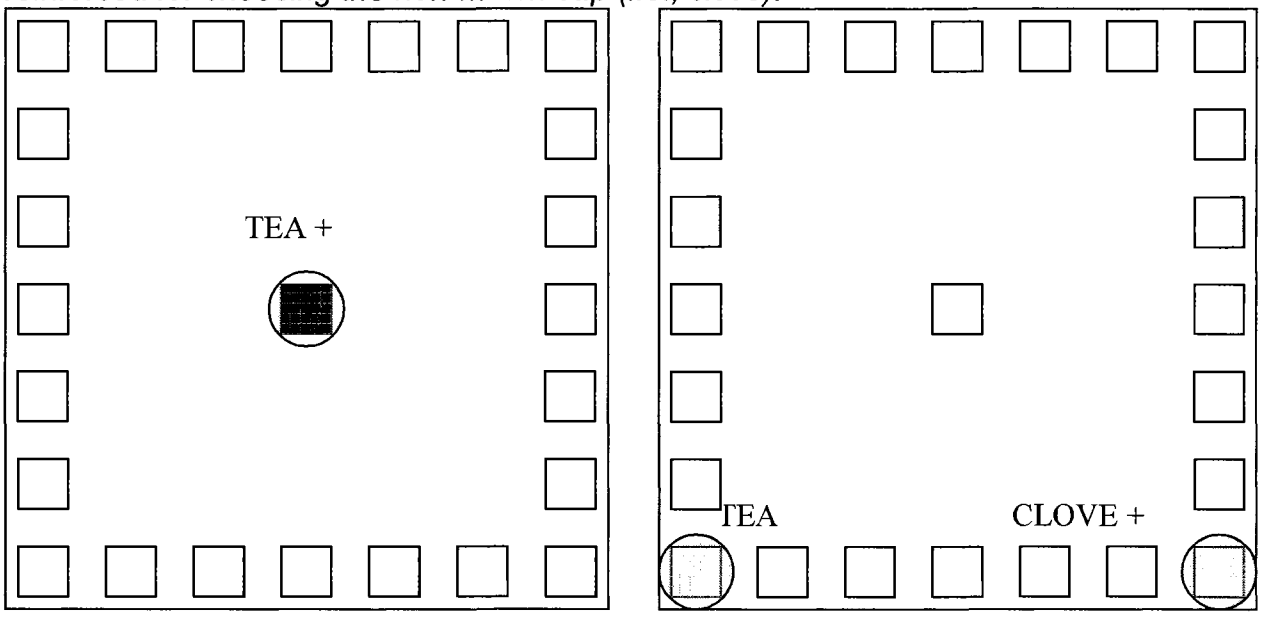
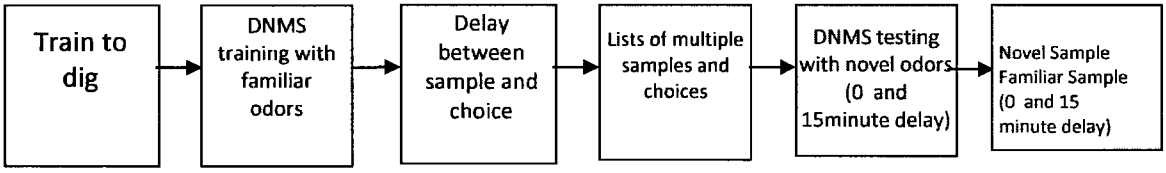


Figure 2: *Typical schedule for DNMS training and task variations. Half the animals began training on delay variations first and half began with list variations. Training with novel odors was also counterbalanced by delay and novelty condition.*



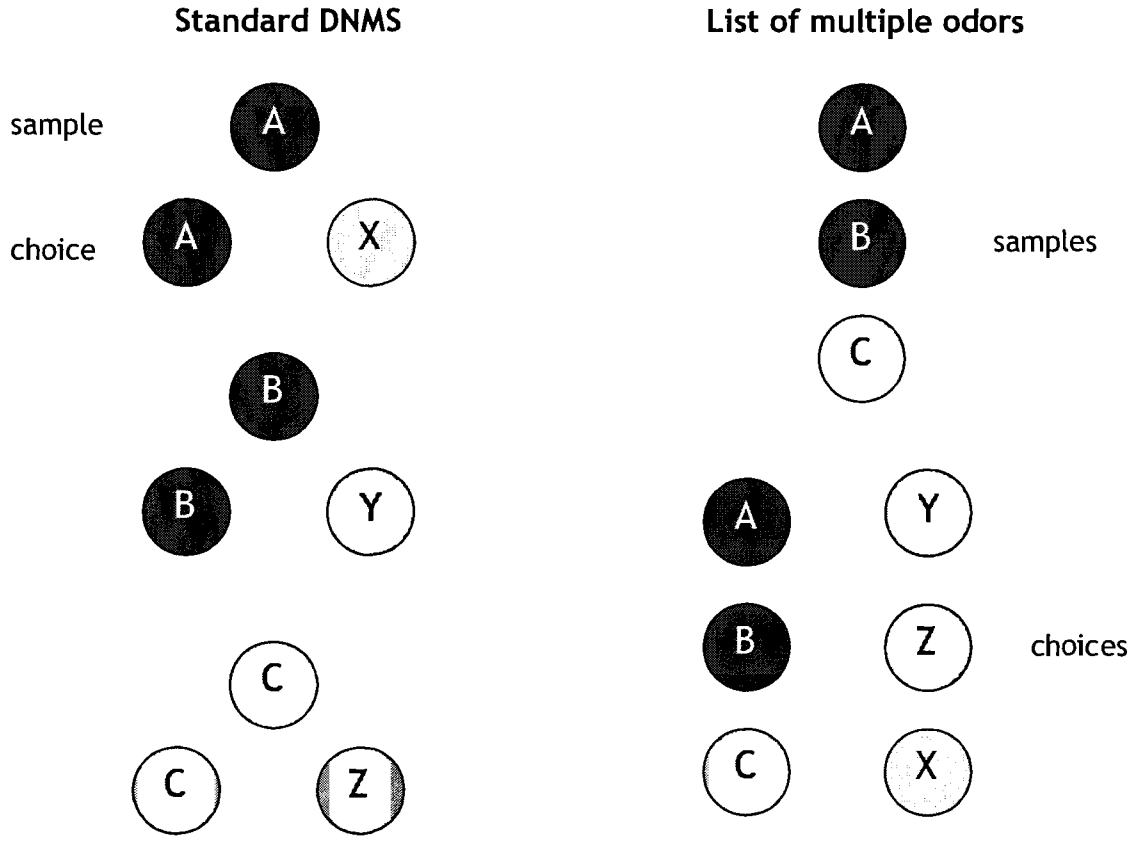
assessing differential mnemonic demand. See Figure 2 for a prototypical schedule of DNMS training task variations.

List length

Animals were required to remember sets of multiple sample stimuli. A series of 2, 3, 5, or 10 sample cups was presented sequentially. Following the presentation of the list of samples, a corresponding list of choices was then presented. Each match odor from the initial list was presented with a non-match that was never used as a sample odor in that session. Animals were exposed to the different list lengths (2, 3, 5, and 10 items) in a counterbalanced order. See Figure 3 for a schematic representation of the list length task variation.

Accurate performance on the list variation of the task requires the subject to maintain representations of multiple stimuli simultaneously. This type of working memory is often assessed in humans using an N-back task (i.e., Cohen et al., 2007; Stern et al., 2001; Mattay et al., 2006; Tsuchida & Fellows, 2008). This task requires participants to monitor sequential presentations of stimuli and either recognize or recall which stimulus was seen N items back. This type of memory has been shown to be associated with prefrontal activity when the items to be remembered are highly familiar (Stern et al., 2001). Moreover, performance in this task is sensitive to prefrontal damage in humans (Tsuchida and Fellows, 2008), and to be impaired with age (Mattay et al., 2006). Typically human subjects are tested on 1- or 2-back versions of the N-back task; this is similar to

Figure 3. *Schematic comparing the standard DNMS task (left column) to DNMS for a list of multiple stimuli, in this case using three odors (right column). In the standard DNMS task, one sample phase (i.e., odor A) is followed by one choice phase containing the incorrect match cup and the correct non-match cup (i.e., odor A vs. odor X, left column, top). Subsequent trials also each contain one sample phase followed by one choice phase (left column, middle and bottom). In the list variation of the task, the sample phase consists of sequential presentation of three sample odors (i.e., odors A, B, and C, right column, top). The subject then receives three sequential choice phases, each containing one incorrect match in the order that it appeared in the initial sample phase and one correct non-match choice (i.e., odor A vs. odor Y, odor B vs. odor Z, and odor C vs. odor X, right column, bottom).*



the 2-item and 3-item lists in the DNMS task, where rats were essentially required to recognize odors that they were exposed to either 1 or 2 trials previously. The 5- and 10-item further increased the cognitive demand and examined whether memory for these longer lists was impaired by the prefrontal lesion.

Delay

To test for impairments in how long a particular stimulus can be held online, the delay between the presentation of the sample and the choice cups was varied between 3, 15, 30, and 60 minutes with the order of delay presentations counterbalanced. The delay sessions consisted of five trials each. This variation of the DNMS task assessed the effects of cholinergic deafferentation of prefrontal cortex on the ability to hold the representation of one item online for a prolonged period (Dunnett, 1990; Sloan et al., 2006).

This task variation also allowed the opportunity to assess whether any impairments were mnemonic or non-mnemonic. Delay-independent impairments, i.e., equally reduced performance at any delay length, would suggest a problem with attending to or encoding the information. In contrast, delay-dependent impairments, i.e., increasingly impaired performance at longer delay lengths, would suggest a deterioration in stimulus representations over time (Dunnett, 1990).

All novel odors

20 odors that the animal had never been previously exposed to were tested while all other aspects of the DNMS task remained the same. To examine the effect of delay on performance with novel odors, one novel odor set was tested with a 15-minute delay in between the sample and choice phases; a second novel odor set was tested with a minimal (~20 second) delay between the sample and choice phases. Since the rats were initially trained with one of two familiar sets of 20 odors, the set that each subject had not been previously exposed to was used to create the two separate novel sets of 10 odors each; one was used for the 15-minute delay session and the other was used for the 0-minute delay session. Since familiar odors for half the rats were used as the novel odors for the other half, differences in performance could be attributed to novelty and not to specific odors tested.

Novel sample

An odor that the subject had not encountered before during either familiar or novel training was presented as the sample. In the choice phase, the match cup was therefore a clone of the novel sample odor while the nonmatch (correct) response was a familiar odor that the animal had been exposed to extensively in prior training (see Figure 4). One novel sample session was tested at a 15 minute delay, and a second session (using different novel odors) was tested at a minimal (~20 second) delay, referred to as the 0-minute delay. This task variation examined the role of age-related entorhinal impairments in the encoding

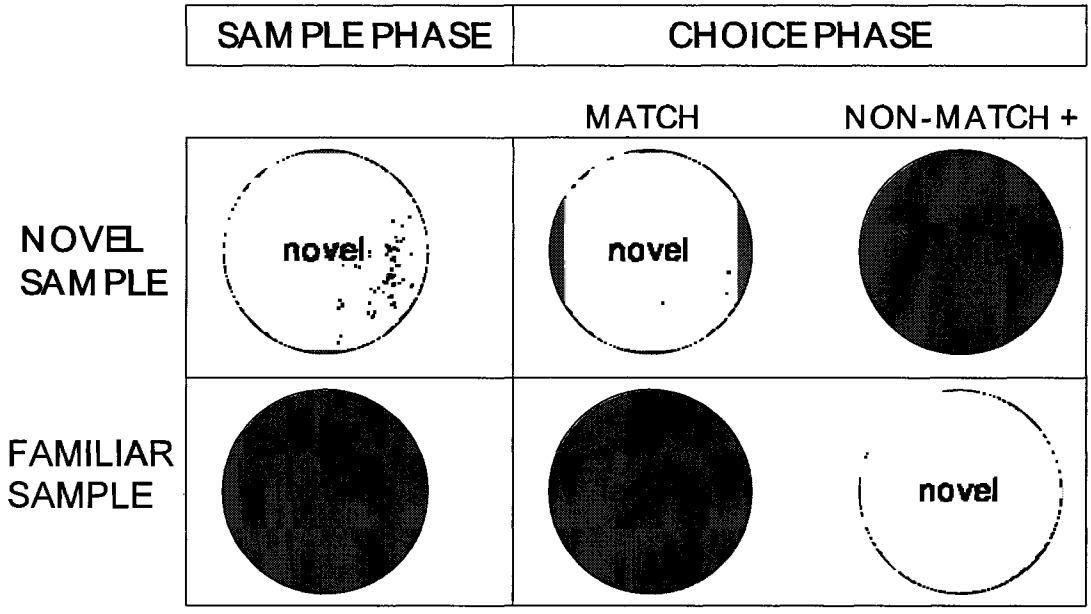
process for novel information. If the subject was impaired at initially encoding the novel sample odor, he would not be able to recognize which of the two choice cups was the correct response even with the ability to recognize the familiar odor. Performance deficits in this variation of the task suggest an inability to encode or maintain a representation of a novel stimulus.

Familiar Sample

A familiar odor used extensively during initial training was presented as the sample cup. The choice phase consisted of the familiar match and a novel nonmatch that the subject had not been exposed to before (see Figure 4). One novel choice session was tested at a 15 minute delay, and a second session (using different novel odors) was tested the 0-minute delay. This task variation targets the role of prefrontal impairments in the retrieval process of familiar information, and the ability to differentiate this information from a novel stimulus. If the subject was able to encode the familiar sample, then during the choice phase he should be able to correctly identify the match cup (i.e., the incorrect response) and choose the nonmatch even without being able to recognize the particular novel odor.

The novel sample 15-minute delay, novel sample 0-minute delay, novel choice 15-minute delay, and novel choice 0-minute delay sessions were presented in a counterbalanced order to all subjects.

Figure 4. *Novel sample and familiar sample testing. Novel sample (top): the animal is exposed to a sample odor he has never previously encountered; the choice phase consists of a clone of that odor and a highly familiar nonmatch. Familiar sample (bottom): a highly familiar odor is the sample; the choice phase consists of a clone of that odor and a correct nonmatch that the animal has never been previously exposed to.*



Dependent Measures and Statistical Analysis

For the standard DNMS task and each task variation the following dependent measures were recorded: overall accuracy, first cup incorrect accuracy, sample response time, choice response time, correct choice response time, and incorrect choice response time. Accuracy was defined as the number of correct responses divided by the number of correct plus incorrect responses. First cup incorrect accuracy was defined as the number of correct responses on trials where the incorrect cup was approached first, divided by correct plus incorrect trials where the incorrect cup was approached first. This measure was recorded to examine the possibility that animals with prefrontal cholinergic lesions were more impaired when they encountered a repetition of the sample odor. As the prefrontal cortex is associated with organizing familiar information, animals with PFC lesions are thought to rely on the EC for encoding the familiar sample. It has been hypothesized that these animals are more impaired when they approach the incorrect cup than when they approach the correct cup first, because cholinergic neurons in the EC respond to the repetition of the sample odor as if it had never been seen before; the animal loses the representation of the original sample (McGaughy et al., 2004).

Sample reaction time was defined as latency to dig in the sample cup during the sample phase of the DNMS task. Choice reaction time was defined as

latency to dig in a cup during the choice phase. This was further broken down into latency to make a correct response or an incorrect response.

For each variation of the DNMS task, a repeated measures ANOVA was performed using SPSS v. 15.0 (SPSS, Chicago, Illinois). For each of the dependent measures, the task variation (i.e., delay length, list length) was entered as the within subjects factor and lesion was entered as the between subjects factor.

Histology

When animals completed all testing, they were anesthetized and transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde in phosphate buffer. Brains were removed and placed in a 30% sucrose solution for 48 hours then sliced into 40 μm coronal sections. Sections were stained for acetylcholinesterase (Tago, Kimura, and Maeda, 1986; McGaughy, Kaiser, & Sarter, 1996). Briefly, sections were placed in phosphate buffer (pH 7.4) containing 0.1% H_2O_2 for 30 minutes to decrease non-specific staining. Sections were then rinsed three times for three minutes each in 0.1 M maleate buffer (pH 6.0), then incubated in a solution of 5 mg acetylthiocholine iodide (Acros Organics, Geel, Belgium), 0.147 g sodium citrate, 0.075 g copper sulfate, and 0.0164 g potassium ferricyanide in 0.1 M maleate buffer for 60 minutes. Sections were rinsed three times for three minutes each in 50 mM Tris buffer (pH 7.6), then incubated in a solution of 0.05 g diaminobenzidine (Sigma, St. Louis, MO)

and 0.375 g nickel ammonium sulfate (Sigma, St. Louis, MO) in 125 ml of 50 mM Tris buffer for 10 minutes. Twelve drops of 0.1% H_2O_2 were added to each 125 ml bath of the diaminobenzidine solution, until cortical layers became visible. Finally, sections were rinsed three times for three minutes each in 5 mM Tris buffer (pH 7.6) and mounted on gelatin-coated slides.

Sections were examined using an Olympus BX51 microscope (Olympus, Melville, NY) at 400x magnification and photographed by a SPOT Insight digital camera (Diagnostic Imaging, Inc., Sterling Heights, MI). Assessment of cholinergic fiber loss was made using a modified grid counting procedure (McGaughy, Kaiser, & Sarter, 1996; McGaughy et al., 2005). A grid measuring 300 by 300 μm was applied to the image of the brain using Image Pro Plus v. 6.0 software (Media Cybernetics, Silver Springs, MD), and the number of fibers that crossed the border of the grid were counted. Fiber counts were obtained in the prelimbic cortex of the mPFC at bregma +4.7, +3.7, +2.7, and +2.5 mm, and in the cingulate cortex at bregma +3.7 and +2.5 mm. To examine possible age-related cholinergic damage in the medial entorhinal cortex (MEC), fiber counts were also obtained in the MEC at bregma -7.8, -7.3, -6.8, and -6.3 mm.

CHAPTER VI

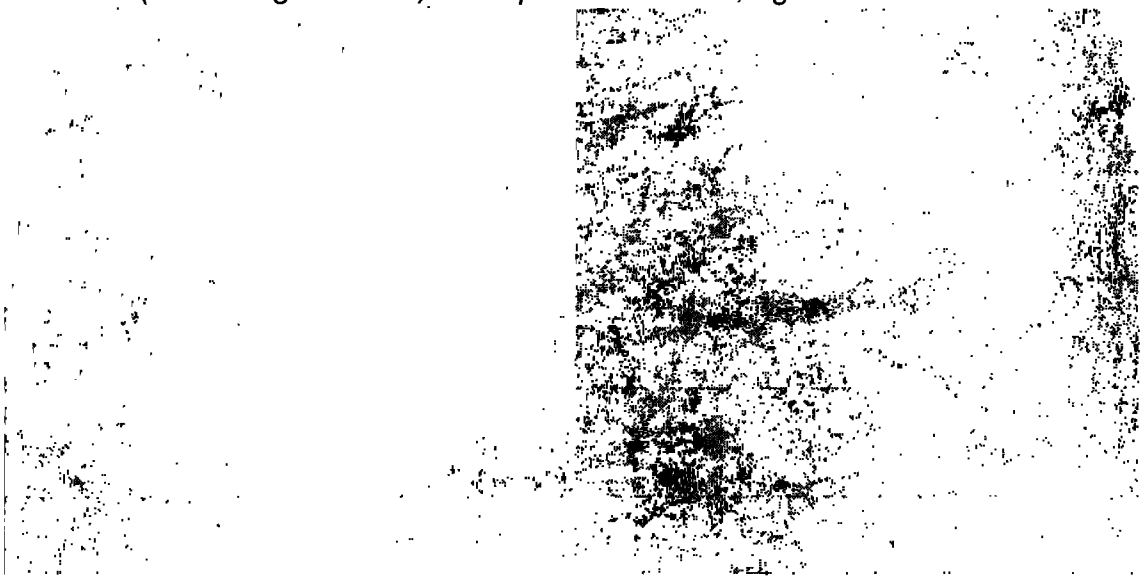
RESULTS

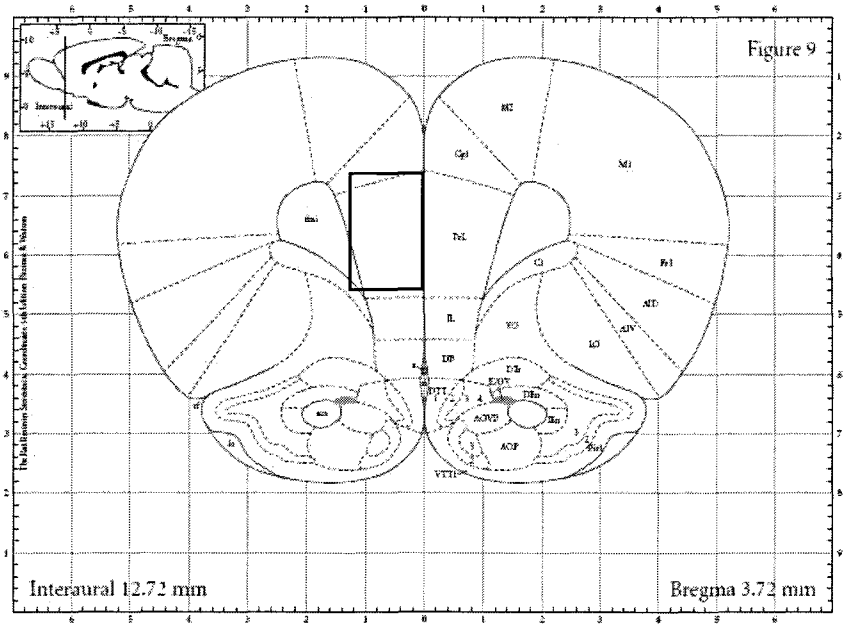
Histology

A repeated measures ANOVA was performed for fiber counts in the prelimbic cortex (PL), with position relative to bregma (+4.7, +3.7, +2.7, +2.5) as the within subjects factor and lesion as the between subjects factor. The lesion effectively produced cholinergic fiber loss in the PL, $F(1,10) = 71.65$, $P < 0.001$. There were no effects of rostral to caudal position, $F(1,10) = 0.55$, $p = 0.64$. Means for number of fibers crossing the grid were sham-lx: 120.6 ± 5.2, pACh-lx: 73.9 ± 2.0, indicating a 38.8% fiber loss in the pACh-lx animals relative to sham-lx animals (see Figure 5).

A separate repeated measures ANOVA was performed for the cingulate cortex (CG), with position relative to bregma (+3.7, +2.5) as the within subjects factor and lesion as the between subjects factor. There was concurrent cholinergic fiber loss in the CG region, $F(1,10) = 49.82$, $p < 0.001$. There were no effects of rostral to caudal position, $F(1,10) = 1.57$, $p = 0.24$. Mean fiber counts were 123.9 ± 5.3 for sham-lx animals and 62.9 ± 6.8 for pACh-lx animals (49.3% fiber loss in pACh-lx animals).

Figure 5. *Sections stained for AChE+ fibers showing prelimbic cortex at Bregma +3.7 mm (400x magnification). Left: pACh-lx animal; right: sham-lx animal.*





From Paxinos & Watson, 2004

The entorhinal cortex shares cortical connections with the medial prefrontal cortex (Insausti, Herrero, & Witter, 1997) and non-specific prefrontal lesions have been shown to alter neuronal firing in the medial temporal lobes (Zironi, Iacovelli, Aicardi, Liu, & Bilkey, 2001). Therefore, to determine if the prefrontal lesion had differential effects on cholinergic fibers in the medial entorhinal cortex (MEC), a repeated measures ANOVA was performed for the MEC with position relative to bregma (-6.3, -6.8, -7.3, -7.8) as the within subjects factor and lesion as the between subjects factor. There was no effect of the prefrontal lesion on fiber counts in the MEC, $F(1,10) = 1.76$, $p = 0.21$, and no effects of rostral to caudal position, $F(1,10) = 1.17$, $p = 0.34$. Because there were no group differences, the aged animals' data were combined and compared with that of young rats with and without 192-IgG-Saporin induced cholinergic lesions of the medial entorhinal cortex (from Newman & McGaughy, 2009).

A repeated measures ANOVA for the MEC with position relative to bregma (-6.3, -6.8, -7.3, -7.8) as the within subjects factor and lesion (young sham-lx, young MEC-lx, and aged) revealed group differences, $F(2,20) = 37.42$, $p < 0.001$. T-tests demonstrated that young sham-lx animals had significantly more fibers in the MEC than young MEC-lx ($t(10) = 9.56$, $p < 0.001$) and aged animals ($t(16) = 7.33$, $p < 0.001$). However, there was no significant difference between MEC fiber counts in young MEC-lx and aged animals ($t(16) = -.190$, $p = 0.85$); see Figure 6. There was a significant effect of rostral to caudal position, $F(3,60) = 4.48$, $p = 0.01$ and a significant position x lesion interaction, $F(6,60) = 4.21$, $p = 0.001$. Young MEC-lx subjects had the greatest loss at bregma -7.8

that decreased slightly in the more rostral positions as indicated by significant differences between each position, all $t(5) > 3.8$, all $ps < .02$, except between bregma-7.8 and -7.3, where a trend still remained for greater loss in the more caudal position, $t(5) = 2.29$, $p = 0.07$. Aged subjects had no significant differences between any of the positions, all $t(10) < 2.00$, all $ps > 0.14$, except between bregma -7.8 and -6.8, where there was a trend for greater fiber loss in the caudal region $t(10) = 2.07$, $p = 0.07$. Two subjects (one sham-lx, one pACh-lx) that were included in behavioral analyses were excluded from histological analyses due to poor staining.

Memory for Familiar Odors

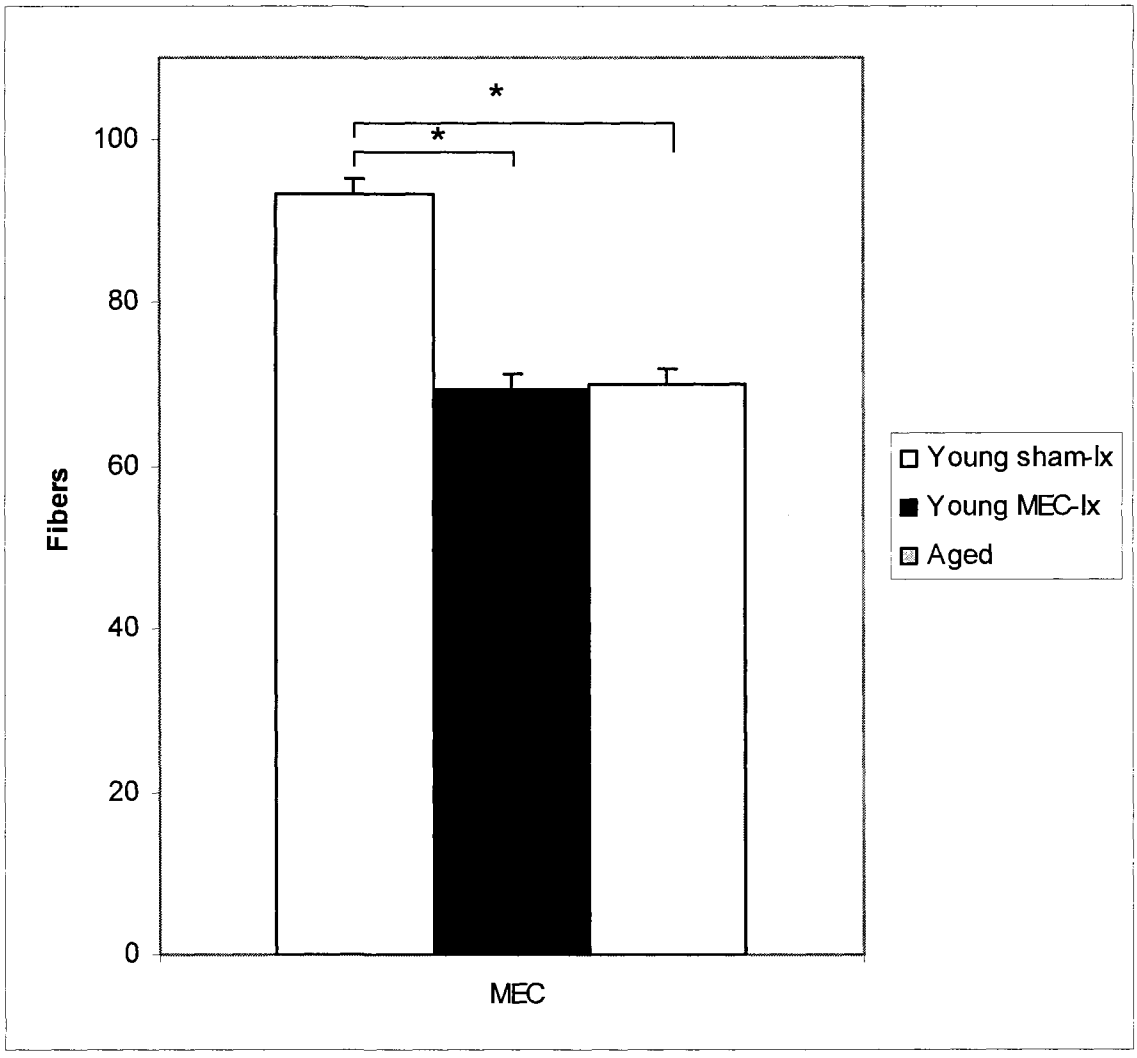
Acquisition

There were no differences between sham-lx and pACh-lx animals in the number of days required to achieve criterion performance at the standard DNMS task, $t(12) = -0.55$, $p = 0.59$ (sham-lx: 14.7 $\pm$ 1.3 days, pACh-lx: 17.4 $\pm$ 4.2 days).

Standard DNMS task

The 2 days prior to the first test of differential mnemonic demand were analyzed using a repeated measures ANOVA with baseline day (first or second) as the within subjects factor and lesion as the between subjects factor. All animals were required to perform at >80% accuracy before the first test of differential mnemonic demand. There were no significant group differences

Figure 6. *Raw fiber counts for medial entorhinal cortex for young sham-lx (white bars), young MEC-lx (black bars) and aged rats (yellow bars; bars show MEAN + SEM). There was no significant difference in raw counts between young MEC-lx animals and the aged subjects in the current study.*



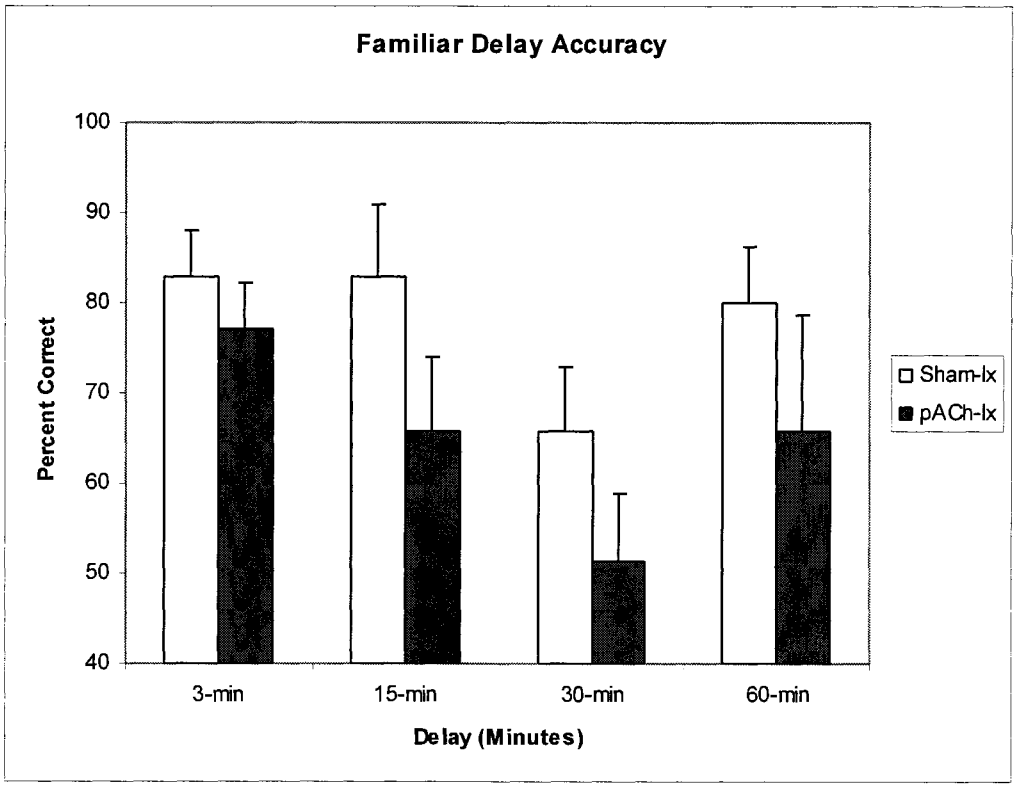
between the two days of standard DNMS testing in terms of overall accuracy ($F(1, 12) = .05$; $p = 0.83$), accuracy on first cup incorrect trials ($F(1,12) = 0.03$; $p = 0.86$), sample response latency ($F(1,16) = 0.73$; $p = 0.41$), or choice response latency ($F(1,16) = 0.27$; $p = 0.62$).

Delay

A repeated measures ANOVA with four levels of delay length (3, 15, 30, 60 minutes) as the within subjects factor and lesion as the between subjects factor revealed a strong trend for an effect of lesion on overall accuracy, $F(1,12) = 4.23$, $p = 0.06$. Overall, sham-lx animals were more accurate ($77.9 \pm 4.4\%$) than pACh-lx animals ($65.0 \pm 4.4\%$). There was a significant effect of delay length on overall accuracy, $F(3,36) = 2.89$, $p = 0.049$; however, this did not interact with lesion, $F(3,36) = 0.21$, $p = 0.89$. Planned comparisons revealed that although pACh-lx animals were less accurate overall than sham-lx animals, there were no significant differences between groups at any individual delay, all $t(12) < 1.50$, all $ps > 0.15$; see Figure 7.

There was a main effect of delay on accuracy for incorrect cup first trials, $F(3,33) = 3.90$, $p = 0.02$, with first cup incorrect accuracy lower for longer delays. One sham-lx animal was excluded from this analysis because he never approached the incorrect cup first during one delay session. However, there was no effect of lesion on first cup incorrect accuracy, $F(1,11) = 0.58$, $p = 0.46$, and no delay x lesion interaction, $F(3,33) = 0.61$, $p = 0.97$. There were no effects of

Figure 7. *Accuracy for sham-lx animals (open bars) and pACh-lx animals (blue bars) with increasing delay (bars show MEAN + SEM). There were no significant group differences between any of the individual delay lengths; however, sham-lx animals were more accurate than pACh-lx animals overall.*



delay on sample response time ($F(3.36) = 0.49$, $p = .64$) or choice response time ($F(3,36) = 1.41$, $p = 0.26$). There were no effects of lesion on sample response time ($F(1,12) = 2.96$, $p = 0.11$) or choice response time ($F(1,12) = 0.01$, $p = 0.93$). There was no delay x lesion interaction on sample or choice reaction time ($F(3,36) = 0.03$, $p = 0.98$, and $F(3,36) = 1.11$, $p = 0.40$, respectively).

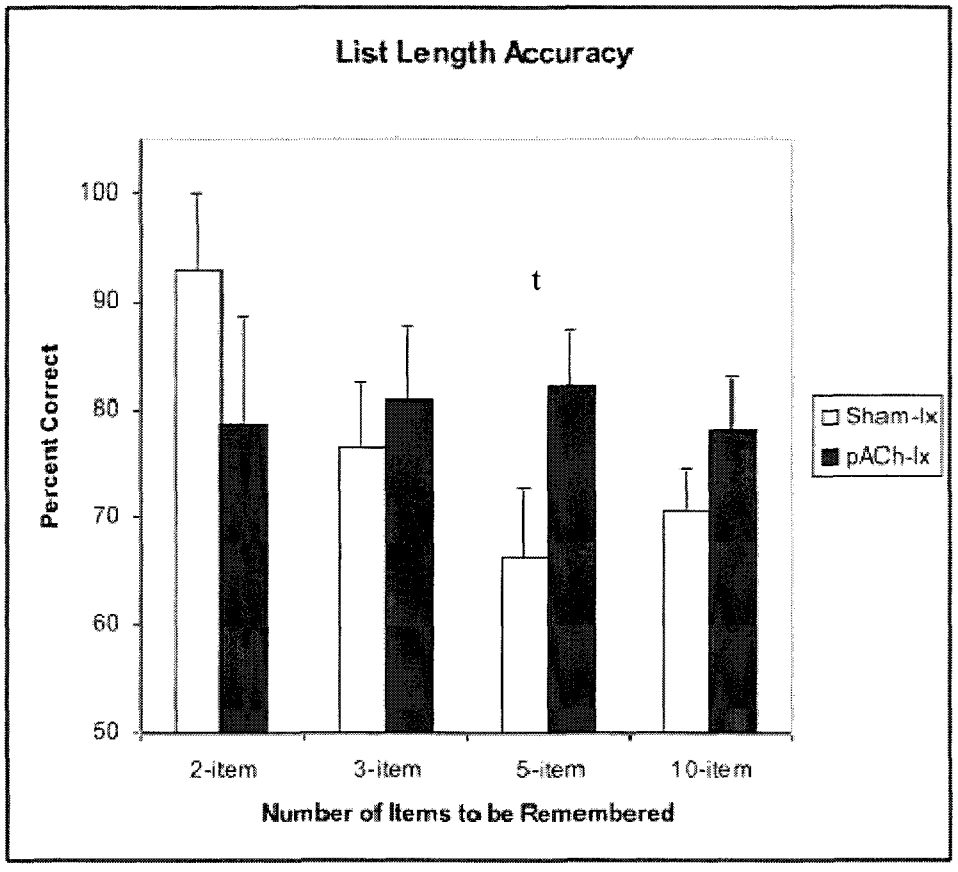
List length

As increasing the number of items to be remembered was expected to increase cognitive demand, a one-way ANOVA was performed with 4 levels of list length (2, 3, 5, 10) for overall accuracy of sham-lx and pACh-lx animals separately to examine whether cognitive capacity was being taxed by this task variation in the way it was intended. This analysis revealed a trend for a main effect of list length on overall accuracy in the sham-lx animals, $F(3,18) = 3.12$, $p = 0.06$, that was not present in the pACh-lx animals, $F(3,18) = 0.08$, $p = 0.95$; see Figure 8.

A repeated measures ANOVA was performed with four levels of list length (2, 3, 5, 10) as the within subjects factor and lesion as the between subjects factor. In terms of overall accuracy, there were no effects of list length ($F(3,36) = 1.24$, $p = 0.31$) or lesion ($F(1,12) = 0.67$, $p = 0.43$), and no list length x lesion interaction ($F(3,36) = 1.72$, $p = 0.19$).

List length did not affect sample response latency ($F(3,36) = 1.46$, $p = 0.25$), or choice response latency ($F(3,36) = 1.35$, $p = 0.26$). Lesion also did not

Figure 8. *Sham-lx animals (open bars) showed a main effect of list length, while pACh-lx animals (blue bars) did not show significant accuracy differences between any of the list lengths (bars show MEAN + SEM). Planned comparisons revealed a trend for pACh-lx animals to be more accurate at the 5-item list, t(13)= 1.92, p = 0.08.*



t = trend; p = 0.08

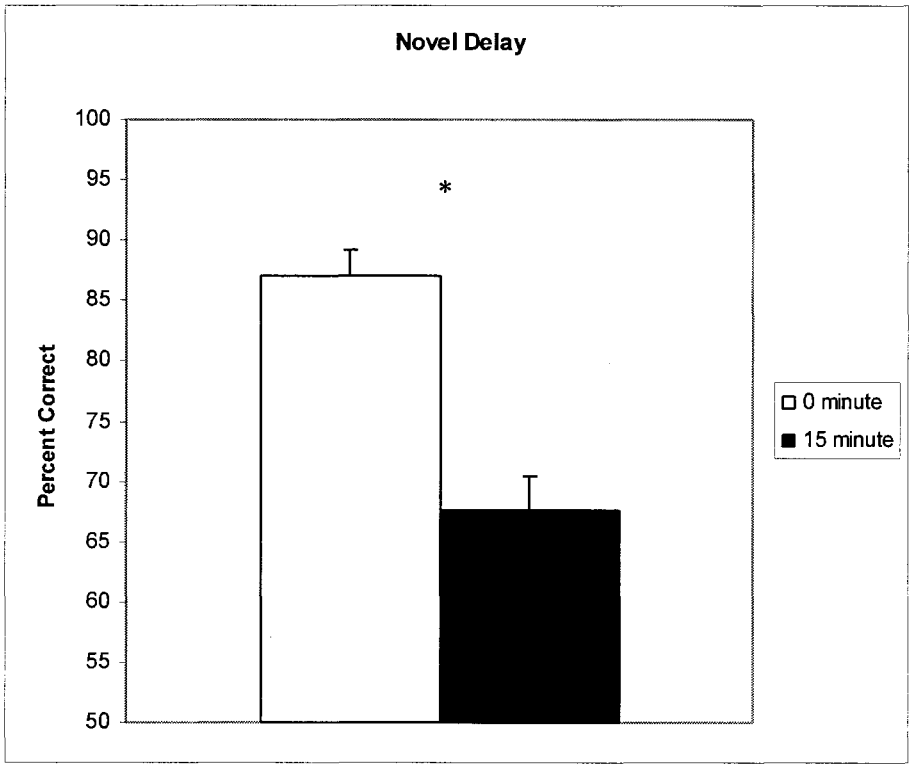
affect sample response latency ($F(1,12) = 0.09$, $p = 0.77$) or choice response latency ($F(1,12) = 1.51$, $p = 0.24$), and there was no delay x lesion interaction on either latency measure (both $F(3,36) < 1.00$, both ps > 0.40). Because 5 subjects (4 sham-lx, 1 pACh-lx) never approached the incorrect cup first during the 2-item list, there were not enough cases to perform an accurate analysis of accuracy for first cup incorrect trials.

Memory for Novel Odors

Subjects were exposed to 3 conditions involving novel odors: tests with all novel odors (both sample and correct non-match), novel sample with familiar non-match choice, and familiar sample with novel non-match choice. Each of these three variations was tested at both a 0-minute delay and a 15-minute delay.

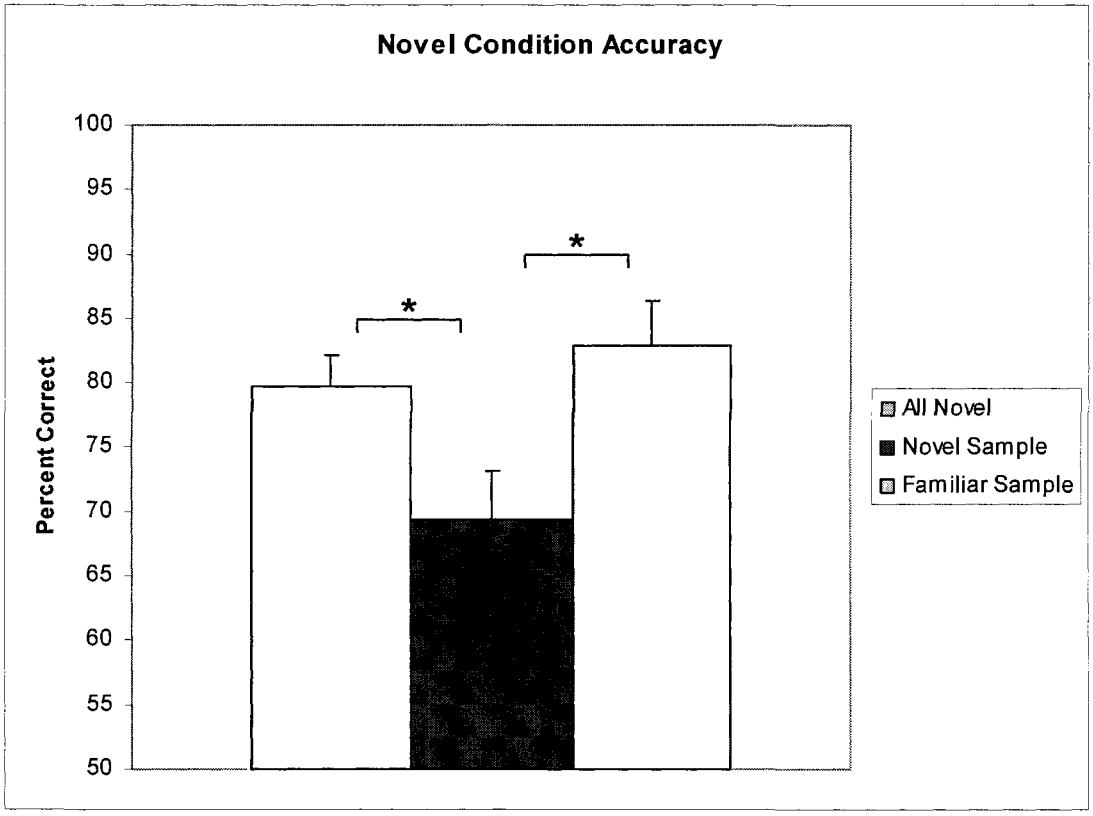
A repeated measures ANOVA with three levels of novelty (all novel, novel sample, familiar sample) and two levels of delay (0 minute, 15 minute) as within subjects factors and lesion as the between subjects factor revealed main effects of both delay ($F(1,12) = 37.97$, $p < 0.001$) and novelty ($F(2,24) = 4.66$, $p = 0.02$) on overall accuracy, but no interaction between these factors, $F(2,24) = 0.10$, $p = 0.91$. There was no effect of lesion on accuracy, $F(1,12) = 1.84$, $p = 0.20$. Subjects were more accurate at sessions with the 0-minute delay than at sessions with the 15-minute delay, $t(13) = 6.25$, $p < 0.001$; see Figure 9.

Figure 9. *Effect of delay collapsed across group (pACh-lx and sham-lx) and novelty condition. 0-minute delay (open bar, MEAN + SEM): 87.0 ± 2.2%, 15-minute delay (black bar, MEAN + SEM): 67.6 ± 2.8%.*



*p < 0.001

Figure 10. *Effect of novelty condition collapsed across group (pACh-lx and sham-lx) and across 0-minute and 15 minute delay. Bars show MEAN + SEM. Subjects were less accurate at the novel sample condition (69.3 ± 3.8%) than at the all novel condition (79.8 ± 2.3%) or the familiar sample condition (82.9 ± 3.5%).*



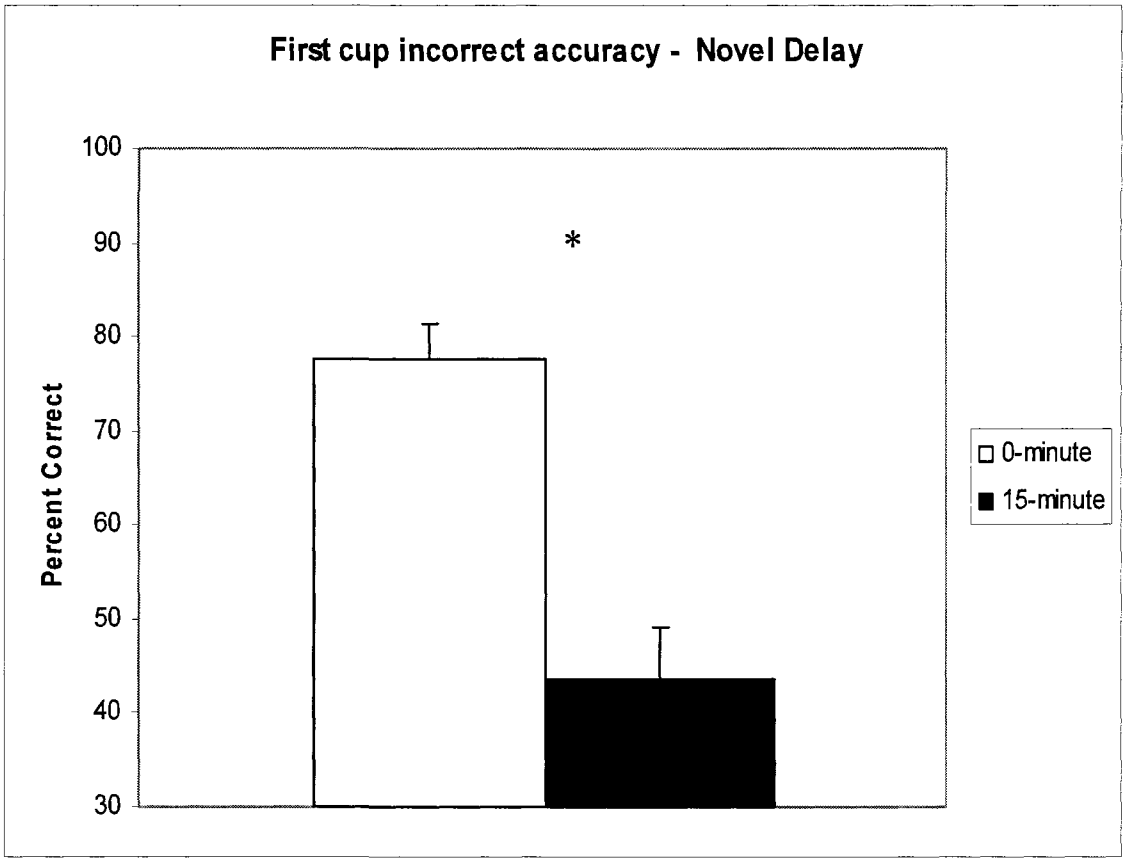
*p < 0.05

Subjects were less accurate at the novel sample condition than the familiar sample condition, $t(13) = 2.51$, $p = 0.03$. Subjects were also less accurate at the novel sample condition than at the all novel condition, $t(13) = 2.25$, $p = 0.04$. There was not a significant accuracy difference between the familiar sample and the all novel conditions, $t(13) = 0.86$, $p = 0.41$; see Figure 10.

For accuracy on first cup incorrect trials, there was also a significant effect of both delay ($F(2,20) = 23.87$, $p < 0.001$) and novelty ($F(2,20) = 5.21$, $p = 0.02$) on incorrect cup first accuracy, but no interaction between these factors, $F(2,20) = 0.003$, $p = 0.99$. There was no effect of lesion on first cup incorrect accuracy, $F(1,10) = 2.07$, $p = 0.18$. Two pACh-lx animals were not included in this analysis because they never approached the incorrect cup first during the 0-minute novel choice session. When novelty conditions were combined, first cup incorrect accuracy was greater at the 0-minute delay than at the 15-minute delay, $t(13) = 4.98$, $p < 0.001$; see Figure 11.

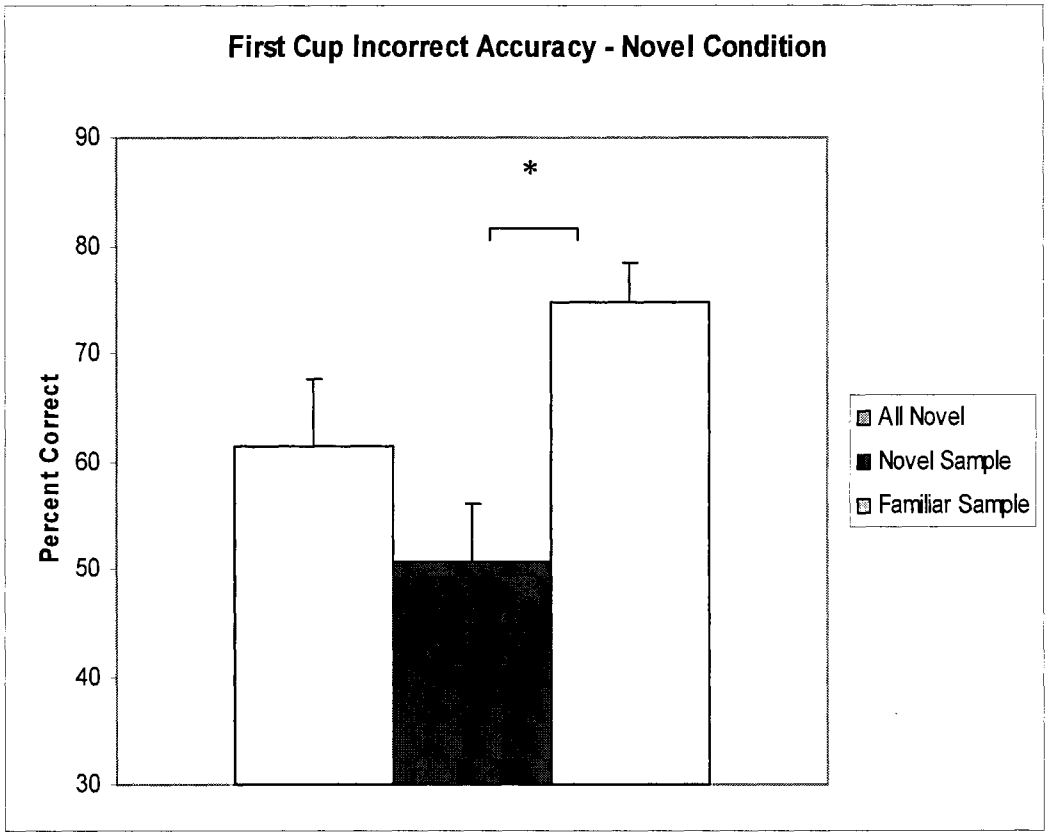
Similar to the pattern found with overall accuracy, first cup incorrect accuracy was greater for familiar sample sessions than for novel sample sessions when collapsed across delay, $t(13) = 3.67$, $p = 0.004$. First cup incorrect accuracy in the all novel condition did not differ from the familiar sample condition ($t(13) = 1.72$, $p = 0.11$). In contrast to overall accuracy, first cup incorrect accuracy during the all novel condition did not differ significantly from the novel sample condition ($t(13) = 1.48$, $p = 0.16$); see Figure 12.

Figure 11. *First cup incorrect accuracy collapsed across group (pACh-lx and sham-lx) and novelty condition. All animals were less impaired at the 0-minute delay (open bar, MEAN + SEM: 77.6 ± 3.7%) than at the 15-minute delay (black bar, MEAN + SEM: 43.6 ± 5.5%).*



*p < 0.001

Figure 12. *Incorrect cup first accuracy during 3 novelty conditions collapsed across delay. Subjects were less accurate at the novel sample condition (50.6 ± 5.4%) than at the familiar sample condition (74.7 ± 3.6%). Accuracy on the all novel condition (61.4 ± 6.3) did not significantly differ from either of the other two conditions.*



*p < .01

Overall accuracy for the all novel condition was significantly greater than for the novel sample condition, but accuracy for first cup incorrect trials did not differ between these two conditions. Therefore a posthoc analysis was performed to examine if this difference could have been mediated by the number of trials where the rat approached the incorrect cup first. There was a significant effect of novelty condition on raw number of first cup incorrect trials, $F(2,24) = 8.45$, $p = 0.002$. Rats approached the incorrect cup first significantly more often during the novel sample sessions ($3.1 \pm .14$ times) than during the all novel sessions ($2.5 \pm .21$ times), $t(13) = 2.52$, $p = 0.03$, and than during the familiar sample sessions ($2.3 \pm .17$ times), $t(13) = 3.56$, $p = 0.003$.

There was an effect of delay length on reaction time, $F(2,24) = 5.14$, $p = 0.04$, where subjects responded faster on sessions with a 15-minute delay (12.15 ± 1.4 seconds) than on sessions with a 0-minute delay (15.52 ± 2.11 seconds). This effect was not mediated by correct choice reaction times (effect of delay: $F(2,24) = 0.80$; $p = 0.39$). As every animal except one had at least one session where they made no incorrect responses, there were not enough cases for an accurate analysis of incorrect response latency. There was a trend for an effect of novelty on choice reaction time, $F(2,24) = 2.70$, $p = 0.09$, where subjects were faster at responding to choices during all novel sessions (11.54 ± 1.4 seconds) than novel sample or novel choice sessions (14.59 ± 2.01 and 15.39 ± 2.23 seconds, respectively).

There were no effects of delay on sample reaction time, $F(2,24) = 0.16$, $p = 0.70$. However, there was a trend for an effect of novelty on sample reaction

time, $F(2,24) = 3.10$, $p = 0.06$, where subjects took longer to respond to samples on novel sample trials (4.10 $\pm$ .72 seconds) than to respond to samples on all novel trials or novel choice trials (2.81 $\pm$ .46 and 3.09 $\pm$ .53 seconds, respectively).

CHAPTER VII

DISCUSSION

This experiment evaluated aged rats with cholinergic lesions of the prelimbic cortex on a delayed non-match to sample task of working memory. Task variations (increasing the delay between the sample and choice phases and increasing the number of stimuli to be remembered at once) were introduced that aimed to increase cognitive demand. Specifically, these variations aimed to tax the prefrontal cortex, which is thought to mediate memory for familiar stimuli over a delay period as well as organize multiple familiar stimuli when there is a high risk of proactive interference (McGaughy et al., 2004, 2005; Schon et al., 2004, 2005; Petrides, 1995; 2000; Dunnett, 1990; 1992).

There was no difference between sham-lx and pACh-lx animals at the standard DNMS task, suggesting that the effects of the pACh lesion could only be differentiated when the cognitive demands of the task were increased. Subjects' performance over increasing delays confirmed the prediction that pACh-lx animals would be impaired at prefrontally-mediated variations of the DNMS task. Accuracy in both groups decreased as the delay period between the sample and the choice phase was increased. The pACh-lx animals were less accurate overall, suggesting a global performance deficit not dependent on delay length.

Previous research has suggested that delay-independent impairments suggest problems with non-mnemonic processes impeding the subject's ability to encode the original stimulus, such as motivation, attention, and encoding (Dunnett, 1990; Müller & Knight, 2006). Aging has been shown to impact attentional abilities and this has been associated with altered cholinergic functioning (Jones, Barnes, Kirkby, & Higgins, 1995; McGaughy & Sarter, 1995; Moore, Dudchenko, Bruno, & Sarter, 1992). Moore et al. (1992) demonstrated that 18-month old rats were impaired at detecting short light signal presentations in a simple reaction time task. Administration of scopolamine produced similar impairments at signal detection in young rats, suggesting dysfunction of the cholinergic system as a reason for aged rats' impairments (McGaughy & Sarter, 1995; Moore et al., 1992; Jones et al., 1995). Based on these findings, it is likely that aged animals in the current study had attentional impairments that decreased their ability to encode the sample stimulus during tasks where the cognitive demands were increased.

An alternative explanation is that the delay-independent effect could be due to concurrent damage to the anterior cingulate cortex, as has been suggested in several studies by Dunnett and colleagues (Dunnett, 1990; Dunnett, Wareham, & Torres, 1990; Sloan et al., 2006). Dunnett suggested that damage restricted to the more rostral areas of the mPFC induces delay-dependent impairments, while damage that extends caudally and into the anterior cingulate cortex, as occurred in the current study, may cause non-specific delay-independent impairments for memory over delays.

It was expected that pACh-lx animals would be impaired relative to sham-lx animals at memory for multiple stimuli. However, this hypothesis was not confirmed as there were no group differences in memory for lists. The strong trend for sham-lx animals to show list-length dependent impairments indicates that cognitive demands were being increased by the longer lists lengths, however, this method of increasing memory load was not sensitive to the effects of the prefrontal lesion. The lack of the pACh-lx animals to show accuracy deficits with increasing list lengths suggests they were actually less sensitive to the effects of proactive interference during this task variation.

It is important to note that because the 2, 3, and 5 item lists did not contain overlapping odor stimuli, they were run in one day. The majority of the subjects in the current study received the 5-item list last (although order of list length presentations was counterbalanced, the data from four subjects who did not receive the 5-item list last will not be presented here). The finding that performance across the different list lengths run in one day was more equivalent for pACh-lx animals supports their reduced sensitivity to proactive interference. In the sham-lx animals, performance at lists they were exposed to later in one session may have been impaired by proactive interference from earlier list lengths that were no longer relevant. This is further supported by the trend for pACh-lx animals to be more accurate than sham-lx animals at the 5-item list.

It was hypothesized that animals in the current study would show age-related deficits at memory for novel information. Aging is associated with reduced cholinergic neurons particularly in the entorhinal cortex (Hyman et al.,

1984; Gomez-Isla et al., 1996; Baxter et al., 1999), which mediates memory for novel information (McGaughy et al., 2005; 2007). It is thought that in response to exposure to novel information, acetylcholine neurons show sustained spiking activity that allow the stimulus representation to remain online and be encoded into memory (McGaughy et al., 2005; 2007). Therefore, it was hypothesized that aged animals in the current study would be impaired at sessions requiring them to encode novel sample odors, relative to sessions with familiar samples but novel non-match choices. This hypothesis was confirmed, as animals were significantly impaired at novel sample sessions relative to familiar sample sessions.

Due to the projections between the entorhinal cortex and medial prefrontal cortex (Insausti, Herrero, & Witter, 1997; Zironi et al., 2001), it was possible that the pACh lesion in the current study could have differentially altered the entorhinal cortex and thus memory for novel odors. However, a lack of group differences between the sham-lx and pACh-lx groups in accuracy for novel odors indicated that the pACh-lx did not differentially affect performance for novel odors. This was supported by the fact that there were no differences in raw numbers of cholinergic fibers in the MEC between the sham-lx and pACh-lx groups. However, the aged animals as a group had raw numbers of cholinergic fibers in the MEC similar to young animals with 192-IgG Saporin- induced lesions of the MEC (Newman & McGaughy, 2009), suggesting that these animals do have a substantial amount of cholinergic fiber loss in the regions known to mediate memory for novel information.

All subjects were less accurate on all novel conditions with 15-minute delays relative to the same sessions with 0-minute delays, indicating that the 15-minute delay condition is more cognitively demanding as it was intended. An additional possibility for why animals were less accurate at the 15-minute condition is the length of time they spent exploring the choice cups. Animals explored the choice cups for an average of three seconds longer during the sessions with 0-minute delays (15.5 $\pm$ 2.1 vs. 12.1 $\pm$ 1.4 seconds); therefore, it is possible that decreased exploration on choice trials, perhaps as a result of having waited through the 15 minute delay, may have contributed to the accuracy impairments seen in these sessions.

Overall, subjects were less accurate on sessions with novel samples than on sessions with familiar samples (70% vs. 83%, respectively). Subjects also were less accurate on sessions with all novel odors relative to sessions with novel samples, an interesting finding considering that both sessions require the encoding of a novel sample. However, a possible reason for this difference is exposed when the first cup incorrect accuracy is analyzed separately. On first cup incorrect trials, there is no significant accuracy difference between the novel sample and the all novel conditions, but both are significantly less accurate than first cup incorrect accuracy on familiar sample trials. Animals approached the incorrect cup first more often on novel sample trials (3.1 $\pm$ .14 times) than on all novel or familiar sample (2.5 $\pm$ 2.1 and 2.3 $\pm$ .17 times, respectively). Therefore, even though subjects were equally impaired at first cup incorrect trials on both conditions that required the encoding of novel samples, their tendency to

approach the correct cup first during the all novel sessions could have contributed to why overall accuracy was better on these trials.

However, subjects still performed with relatively high accuracy at the all novel variation of the DNMS task. The current subjects performed at 70% accuracy on the 15-minute delay session with all novel odors, in contrast to previous studies where young subjects with cholinergic lesions of the medial entorhinal cortex performed at 40% accuracy for all novel odors with the same delay (McGaughy et al., 2005). Aged animals' performance for all novel odors in the current study was even higher (88%) at the 0-minute delay condition.

This raises the issue of why animals in the current study are less impaired at memory for novel odors than young, MEC-lesioned animals from previous studies (McGaughy et al., 2005; McGaughy et al., 2007). First, because the effects of aging are gradual, animals acquired the task already having lower levels of cholinergic fibers in MEC, in contrast to being trained on the task while intact and then receiving a lesion. Therefore, subjects may have been able to recruit other areas of the brain that were less impaired by aging to perform the task. Extensive behavioral training at a separate operant task throughout the aging process may have enhanced this ability to compensate for impairments. Studies in humans show that aged individuals are able to compensate for age related impairments while not showing behavioral differences in performance (Mattay et al., 2006).

A limitation of the current study is that these data represent a small N (7 sham-Ix, 7 pACh-Ix animals), which could reduce the ability to distinguish

significant differences between groups. Future research testing more subjects on these task variations would reduce this issue. This study confirmed that pACh-lx animals were impaired at prefrontally-mediated variations of the DNMS task, particularly memory for familiar odors over increasing delays. Subjects also demonstrated a separate impairment for performance at sessions with novel samples relative to sessions with familiar samples. This impairment in memory for novel stimuli, which is less pronounced than that seen in young animals with cholinergic lesions of the MEC, still appears to be due to age-related cholinergic depletion in the entorhinal cortex as confirmed by fiber counts. Although performance deficits may have been minimized by animals' compensation due to extensive behavioral training throughout the process of aging, aged subjects still attained the highest levels of accuracy when the delay was minimal and the sample was familiar. Overall, the current study confirmed the role of acetylcholine in the prelimbic cortex in memory for familiar stimuli over delays, and confirmed the role of age-related cholinergic depletion in memory for novel stimuli.

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APPENDIX

UNIVERSITY of NEW HAMPSHIRE

May 24, 2006

McGaughy, Jill
Psychology, Conant Hall
Durham, NH 03824

IACUC #: 060404
Approval Date: 04/28/2006
Review Level: D

Project: The Role of Orexin Neurons in the Prefrontal Cortex in Attention

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category D on Page 4 of the Application for Review of Vertebrate Animal Use in Research or Instruction - *the research potentially involves minor short-term pain, discomfort or distress which will be treated with appropriate anesthetics/analgesics or other assessments.*

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:

1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladi Porsche, UNH Health Services.

If you have any questions, please contact either Roger Wells at 862-2726 or Julie Simpson at 862-2003.

For the IACUC,

Jessica Bolker

Jessica A. Bolker, Ph.D.
Chair

cc: File

Research Conduct and Compliance Services, Office of Sponsored Research, Service Building, 51 College Road, Durham, NH 03824-3585 * Fax: 603-862-3564