EFFECTS OF SELECTIVE LESIONS OF THE NUCLEUS

BASALIS MAGNOCELLULARIS ON WORKING

MEMORY IN RATS

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by

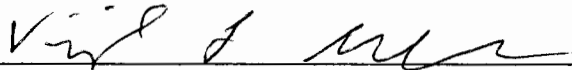Michelle Marie Noble

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# APPROVAL SHEET

The thesis of Michelle Marie Noble, Contribution to the School of Graduate Studies, Indiana State University, Series I, Number 2153, under the title *Effects of Selective Lesions of the Nucleus Basalis Magnocellularis on Working Memory in Rats* is approved as partial fulfillment of the Master of Arts Degree in the amount of six semester hours of graduate credit.
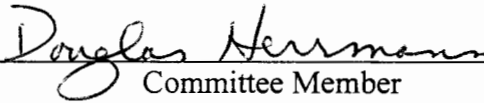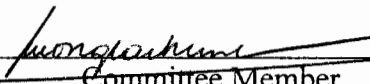
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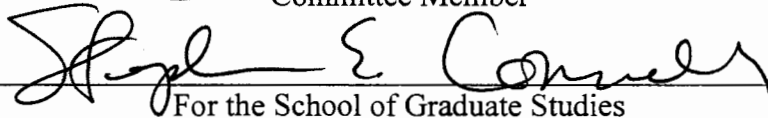
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ABSTRACT

The nucleus basalis magnocellularis (NBM) of the rat brain is analogous to the nucleus basalis of Meynert found in humans. Alzheimer's disease patients have working memory impairments, which may be attributable to damage to the basal nucleus of Meynert. Excitotoxins such as quisqualic and ibotenic acid have been previously used to make lesions of the NBM in research animals. NBM lesions made with ibotenic or quisqualic acid are known to impair working memory. However, in addition to damaging the cholinergic neurons of the NBM, the lesions made by these excitotoxins also destroy cells of other nearby structures, and it is unclear whether the impairments found are due to damage to the NBM or to surrounding non-cholinergic structures. With the recent advent of the highly selective immunotoxin 192 IgG-saporin, it may be possible to determine if lesions involving only the cortically projecting NBM cholinergic neurons impair working memory. The current experiment tests the hypothesis that selective lesions of cholinergic neurons of the NBM impair working memory. To test this hypothesis, a delayed non-matching-to-position-task was used as a test for working memory. Results of this experiment provide novel evidence of the involvement of the cholinergic neurons of the NBM in working memory and will contribute to our understanding of the cognitive impairments seen in Alzheimer's disease.

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Chapter 1

INTRODUCTION

As people age, forgetting becomes more common. However, about 40% of 80-85 year olds in the human population are afflicted with Alzheimer's disease (Harrell, 1991). There are typically three phases in this type of dementia. The first phase consists of memory loss and changes in personality. During this phase, patients' social skills are relatively intact. Diagnosis for dementia is rarely made at this stage, because these impairments are attributed to normal aging processes. The impairments characteristic of the first phase gradually worsen, leading to problems in life skills. The second phase is characterized by loss of memory for familiar people, places, and items. Patients may forget where they are or where they live. It is during this phase that patients, their doctors, and families start to believe that the symptoms are problematic. Diagnosis at this stage is very good. The third phase is characterized by a severe worsening of impairments. Usually at this stage, the patients' family relies on home care or nursing homes for the care of the afflicted family member. Alzheimer's patients eventually end up in the fetal position, have both bladder and bowel incontinence, and are unable to eat or feed themselves. Currently, prognosis is poor for these patients.

Cognitive Impairments in Alzheimer's Disease. Dementia of the Alzheimer's type is the most common form of dementia (Harrell, 1991; Becker, Bajulaiye, & Smith,

1992; Muir, 1997). Deficits commonly found in patients that have Alzheimer's disease include working memory deficits, matching-to-sample deficits, secondary memory deficits, visuospatial short-term memory impairments, and language memory deficits (Harrell, 1991). Working memory, also termed as "recent" memory, is memory for recent events that is maintained only as long as the remembered information is necessary or useful. A common example of working memory in humans occurs when someone looks up a telephone number, dials it, and promptly forgets the number, because it is no longer needed. The ability to store information in working memory is severely impaired in patients with Alzheimer's disease (Baddely, Bressi, Sala, Logie, & Spinnler, 1991; Belleville, Peretz, & Malenfant, 1996). According to Baddeley (1986), working memory has two systems: one for verbal information and one for non-verbal information. These systems are controlled by a Central Executive System (CES). In Alzheimer's disease, the CES seems to be significantly impaired (Becker et al., 1992). AD is characterized by early memory loss as well as impaired CES and forebrain functions (Reid et al., 1996). It has been suggested by Becker (1988), that this CES dysfunction could be accounted for by damage to cholinergic NBM neurons.

Neuropathology of Alzheimer's Disease. Studies of post-mortem brains of Alzheimer's disease patients reveal that the cholinergic neurons in the nucleus basalis of Meynert are damaged (Weinstock, 1997; Herron, Li, & Schweitzer, 1998). Correlated with the destruction of these cholinergic neurons is the lack of neocortical acetylcholine (Ach) normally released by the nucleus basalis neurons (Harrell, 1991; Chiba, Bucci, Holland, & Gallagher, 1995; Bigl & Schliebs, 1998). Typically, the severity of degeneration of the basal forebrain seen in Alzheimer's patients correlates with the

degree or severity found in working memory impairments (Cheng, Ren, & Tang, 1996; Iversen, 1997). In addition to pathology in the basal forebrain, the neocortex also undergoes severe degeneration in Alzheimer's disease. Neurofibrillary tangles, caused by abnormal tau activity, and plaques, caused by excess β-amaloid acitivity, disrupt cortical functioning and thereby compromise a variety of behaviors including cognition (Behrouz, Defossez, Delacourte, & Mazzuca, 1990; Harrell, 1991; Marx, 1992; Iversen, 1997; Lin, LeBlanc, Deacon, & Isacson, 1998; McDonald, Willard, Wenk, & Crawley, 1998). The degree of neurofibrillary tangles present in the frontal lobes is significantly correlated with the degree of dementia observed in Alzheimer's disease (Carlesimo, Fadda, Lorusso, & Caltagirone, 1994).

Animal Models of Alzheimer's Disease. The nucleus basalis magnocellularis (NBM) in the rat brain is analogous to the basal nucleus of Meynert in humans (Robbins, McAlonan, Muir, & Everitt, 1997). Both structures send cholinergic projections to the entire neocortex including the visual and auditory cortices (Wenk, 1997). Damage to the NBM reduces cortical cholinergic activity and impairs cognitive function (for reviews see G. Pepeu, Casamenti, Pedata, Cosi, & Pepeu, 1986; Olton & Wenk, 1987; Riekkinen, Laulumaa, Sirvio, Soininen, & Helkala, 1987; Kesner, 1988; Smith, 1988; Beninger, Wirsching, Jhamandas, & Boegman, 1989; Olton, 1990; Dekker, Connor, & Thal, 1991; Fibiger, 1991; Wenk, 1997). The prevalent hypothesis in animal models of Alzheimer's disease is that cholinergic neuronal activity is necessary for "attention" in certain tasks (Kesner & Johnson, 1995). NBM lesion studies therefore provide an essential model of the cortical cholinergic hypofunction characteristic of Alzheimer's disease (Bartus, 2000). In previous studies NBM lesions were made by infusing excitotoxins such as

quisqualic or ibotenic acid to create animal models. However, these excitotoxins have been found to damage non-cholinergic structures in addition to damaging the cholinergic cells of the NBM, which confounds interpretations of the specific involvement of the cholinergic system in these models (Bigl & Schliebs, 1998).

A recently developed immunotoxin, 192 IgG-saporin, creates highly selective lesions of the NBM cholinergic neurons while sparing non-cholinergic neurons in the vicinity of the lesion (Schliebs, Rossner, & Bigl, 1996; Walsh, Herzog, Gandhi, Stackman, & Wiley, 1996). 192 IgG-saporin is an antibody to the p75 nerve growth factor receptors, which are found in high concentrations on the nicotinic and muscarinic cholinergic neurons of the NBM, but not on surrounding non-cholinergic cells (Baxter, Bucci, Sobel, Williams, Gorman, & Gallagher, 1996; Bigl & Schliebs, 1998; Levin & Simon, 1998; Wiley, 1997). According to Granon, Poucet, Thinus-Blanc, Changeux, & Vidal (1995), injections of neuronal bungarotoxin (NBT) into nicotinic receptors in the prefrontal cortex limbic area impaired attention, and similar impairments have been found in studies involving NBM lesions. Studies using scopolamine demonstrate that muscarinic receptors may be involved in impaired working memory (Dunnett, 1985). 192 IgG-saporin binds to the p75 receptors and disrupts protein synthesis ultimately killing the cell (Waite, Wardlow, & Power, 1999). The selectivity achieved by the use of this immunotoxin has allowed a more accurate determination of the relationship between cholinergic NBM function and behavior. Indeed, several behavioral paradigms that are sensitive to less selective NBM lesions fail to reveal impairments following 192 IgG-saporin infusions into the NBM (Wrenn & Wiley, 1998). An accurate assessment of the NBM's role in cognition, therefore, must rely on the use of the selective immunotoxin

192 IgG-saporin rather than on its less selective predecessors. Nevertheless, earlier NBM lesion research provides a useful framework for understanding the outcome of more current studies using 192 IgG-saporin.

NBM and Working Memory. Previous research in animals with damage to the NBM suggests the existence of a lesion-induced impairment in working memory similar to that seen in Alzheimer's patients (Olton, 1990; Biggan, Beninger, Cockhill, Jhamandas, & Boegman, 1991; Dornan et al. 1996). While the majority of experiments studying potential NBM involvement in working memory have relied on less selective toxins including ibotenic acid, quisqualic acid, and kainic acid, a small number of more recent studies have utilized 192 IgG-saporin in creating lesions of the NBM.

Beninger, Wirscing, Jhamandas, Boegman, and El-Defrawy (1986) found working memory impairments in rats with unilateral injections of kainic or quinolinic acid NBM lesions tested in a delayed-alternation T-maze task. Beninger et al. began each trial with one arm of a T-maze blocked, and the rat had to enter the open arm for food reinforcement. The rat was then transferred to the start box for a 30-second delay. After the delay, the rat was given a choice of which arm to enter, and the rat was rewarded for choosing the side opposite to that most recently visited. During the 30-second delay, the rat had to remember which arm he entered previously; this requirement is argued to depend on working memory. The rats with NBM lesions were unable to perform the task, suggesting impaired working memory.

A previous study using quisqualic and ibotenic acid lesioned rats in a double Y-maze task demonstrated different memory impairments (Beninger, Kuhnemann, Ingles, Jhamandas, & Boegman, 1994). The rats started at one Y in the maze, and they had to go

to the middle of the maze, then move into the 2$^{nd}$ Y, and choose the arm located opposite to the arm they had started in. The working memory component was choosing the correct arm. Upon reaching criterion, rats received bilateral site-specific microinjections of either quisqualic or ibotenic acid. Rats with quisqualate lesions had larger working memory impairment, but both excitotoxins produced working memory impairments.

Another study used quinolinic acid lesioned rats in a T-maze alternation task (Misztal, Skangiel-Kramska, Niewiadomska, & Danysz, 1996). Rats were pretrained to alternate choice of arms in the maze. They received a food pellet if the correct choice was made. Upon reaching criterion, rats were lesioned with intracerebraventricular injections of quinolinic acid or saline injections. Impairments in working memory were found; however, since depletion of cholinergic neurons in the NBM was mild, they believe that working memory impairments may be due to morphological changes in the NBM neurons.

Other researchers have found similar working memory impairments in rats with NBM lesions created by infusing a variety of other neurotoxins including ibotenic and quisqualic acid (Dunnett, 1985; Knowlton, Wenk, Olton, & Coyle, 1985). However, the interpretations of those studies are confounded because these lesions almost certainly damaged more than just the cholinergic neurons of the NBM (Waite & Thai, 1996). Therefore, it is not clear that the behavioral impairments found are caused by lesions to the NBM, or other nearby non-cholinergic structures, or a combination of cholinergic and non-cholinergic neurons in the basal forebrain.

Curzon, Bannon, & Decker (1999) conducted a study intended to study working memory using the selective immunotoxin 192 IgG-saporin infused into the NBM. Sixty-

four male Long-Evans rats were divided into three groups: control, low-dose, and high-dose 192 IgG-saporin groups. All groups were pretrained on a go/no-go task before lesioning. In this task, rats were placed in an operant chamber where a retractable lever was inserted into the chamber, and the rat received continuous reinforcement for lever-pressing during a 20 s interval (i.e., "go" trial). After 20 seconds, the lever was retracted for a period of 5, 20, or 40 seconds. The lever was reintroduced after the intertrial interval, and the rat was not reinforced for lever pressing during the 20 s interval (i.e., "no-go" trial). Every other trial was a no-go trial, and the rats had to remember whether or not they had received reinforcement on the previous trial in order to know whether or not they should press the lever on a given trial; this requirement was the working memory component of the task.

In order to measure go/no-go trial discrimination and working memory performance, Curzon et al. (1999) measured response latency. In this case, response latency was a measure of the time between lever insertion and the first bar press made by the rat following that lever insertion. Rats who learn the go/no-go task have shorter response latencies on go trials and longer response latencies on no-go trials; such differential responding would reflect the animal's understanding that go trials are reinforced and no-go trials are not reinforced (Curzon et al., 1999). Rats that perform well will have short go latencies and long no-go latencies resulting in small ratios (Curzon et al., 1999). Results showed that the control group had good working memory performance at both the 5 and 20 s delays. Both the low-dose 192 IgG-saporin group and the high-dose 192 IgG-saporin group were impaired at all delays (i.e., 5, 20, & 40 s delays), and the differences between these two groups at all delays were not significant.

The data seems to suggest delay dependent impairments, but the statistical test for interaction (group x delay interval) was not significant, perhaps due to the high variability evident in the high-dose 192 IgG-saporin group. Another reason a significant interaction may not have been found may be due to an apparent floor effect in the control group. Forty seconds may have been too long of an intertrial interval placing too great a demand on working memory in this task; none of the groups could perform at the 40 s intertrial interval. If a lower intertrial interval was used, a significant delay dependent effect may have appeared. A delay dependent impairment (i.e., a group x delay interaction) reflecting greater impairments at longer intervals would suggest working memory impairment. Despite the fact that no delay-dependent effect was observed, the Curzon et al. (1999) data suggests that the NBM is involved in some way in solving working memory tasks.

In another study, rats with bilateral 192 IgG-saporin NBM lesions received prior to training in a delayed-alternation T-maze task (i.e., DNMTP) (Wenk, Stoehr, Quintana, Mobley, & Wiley, 1994). They found impaired acquisition during the first two days training. The interpretation of the acquisition impairment is confounded by the fact that during acquisition rats must both learn the DNMTP rule, and they must maintain a working memory representation of the "forced" arm location during the 5 s delay interjected before the "choice" run. Therefore, it is unclear if the NBM lesioned group was unable to learn the rule, unable to maintain the working memory representation, or both. Despite the early acquisition impairment the NBM lesioned group learned to perform the 5 s delay task as well as controls. As a further challenge to working memory capacity, Wenk et al. (1994) increased the delay from 5 to 15 s, although this increase

failed to reveal any impairment in the NBM lesioned group. These data were interpreted to suggest that the NBM is not involved in working memory. However, it may be possible that the NBM is in fact involved in working memory but that the 15 s delay used by Wenk et al. (1994) placed insufficient demands upon the working memory system; it is possible that working memory impairments might be observed if the NBM lesioned animals are subjected to longer working memory retention intervals.

Effects of Selective Lesions of the NBM on Working Memory in Rats. All of the findings previously mentioned suggest that a delayed non-matching-to-sample task with a 0, 60, and 120 s delay may show working memory impairments with saporin lesioned NBM rats. It is important to understand that the working memory task has two components: a go/no-go rule learning component and a delay-dependent component (i.e., the working memory component). Animals cannot solve the working memory task unless they can learn and remember the rule. Because the high-dose 192 IgG-saporin rats in the Curzon et al. (1999) study were equally impaired at all delays, at least according to the statistics, one cannot discern if the rats forgot the rule or if working memory was impaired. If they forgot the rule, they should have equally poor performance across all delays. It seems as if Curzon, Bannon, & Decker's (1999) data suggest an intact memory for the non-matching rule, but an impaired ability to perform the working memory component of the task. At the shortest delay (i.e., 5 s), performance in the high-dose 192 IgG-saporin group was better than chance. The authors concluded that the deficits shown are due to impaired reference memory and/or attention rather than working memory.

The Curzon et al. (1999) study did not include a 0 s delay. To determine whether the rats were impaired in remembering the rule for the task or whether they had delays

from the beginning (suggesting working memory impairments) cannot be determined

unless a 0 s delay is imposed. Butt & Hodge (1995) pretrained rats to discriminate

between two lights. They made ibotenic acid NBM lesions, and they found that the NBM

lesioned group had minimally impaired performance, which suggests that the rats

remember the rule after lesioning. To rule out the problem faced by Curzon et al. (1999)

of whether a working memory impairment, learning, or remembering the rule for the

DNMTP task is responsible for the poor performance of the NBM lesioned rats, the rats

in the current study have been lesioned post training. This ensures that the NBM lesioned

group's poor performance is not due to being unable to learn the task. I proposed that a

delayed non-matching-to-position (DNMTP) task with a 0 s delay would determine

whether NBM saporin lesioned rats were impaired due to whether they showed only

delay impairments (i.e., working memory). I hypothesized that NBM-lesioned rats would

show delay-dependent impairments in working memory performance, with greater

impairment at longer retention intervals. Obtaining the predicted results has provided

critical support for the argument that the NBM is involved in working memory, and

contributes to our understanding of cortical cholinergic involvement in learning and

memory. Results from this type of research may also help us to understand why certain

tasks and not others were found to be impaired with the advent of NBM cholinergic

specific immunotoxin 192 IgG-saporin.

Chapter 2

METHOD

Guidelines for Animal Use. All of the following procedures involving research

animals meet the requirements set by the Society for Neuroscience, the American

Psychological Association, the National Research Council, and the Indiana State

University (ISU) Animal Care and Use Committee.

Animals. Ten male Long-Evans rats (Harlan Sprague-Dawley, Indianapolis, IN)

weighing approximately 250 g were housed individually under a reversed 12-hr light

cycle (lights on at 1800 hrs) for a period of two weeks prior to behavioral and surgical

procedures. Rats were allowed free access to food and water and were handled for five

minutes daily during this time.

Apparatus and Behavioral Testing. All behavioral testing was conducted in a Y-

maze. The maze was constructed out of clear 0.5 cm Plexiglas floors and Plexiglas sides

and lids. The stem and arms of the maze measured 14 cm X 14 cm X 40 cm. A tube (1.5

cm inner diam.) is located at the end of both the right and left arms so that reinforcement

(Cheerios© cereal) may be dropped for food reinforcement. Sliding doors made of clear

Plexiglas were placed at the end of the start arm and at the entrances to both the right and

left arms. The maze was mounted on an opaque sheet of Plexiglas so that the floor of the

maze was not transparent. The maze was placed on a table 1 m above the room floor and posters were located throughout the room to provide distal visual cues.

Beginning one week prior to maze habituation, rats were gradually reduced to and then maintained at approximately 85% of their ad libitum feeding weights. Rats were then habituated to the maze for seven days. Rats then started shaping procedures on day 8, and they continued to receive shaping until day 12. On day 12, rats started training with a 0, 60, and 120 s delayed non-matching-to-position task (DMNTP). The intertrial interval was 60 s, and this occurred in an opaque box (36 cm X 46 cm X 40 cm). Rats received 18 randomly selected forced arm choices with an even number of all three delays. Upon completion of a forced arm choice, both goal arms remained unblocked, and rats were required to choose the opposite goal arm that they were forced to choose previously. High levels of working memory performance were reflected by greater percentages correct on non-matching trials (reinforced trials) and smaller percentages correct on matching trials (non-reinforced trials). Upon entering a goal arm, the gate was closed and the rat remained in the arm for 20 s. Upon completion of 30 days (i.e., 10 blocks) of testing, rats were divided into control and lesion groups that were matched according to the average percentage correct across all delays on the final block of preoperative testing.

Surgery. Rats received bilateral lesions of the NBM created by injecting 192 IgG-saporin, or sham lesions using sterile phosphate-buffered saline injected into the NBM (n=10) (Baxter, Bucci, Gorman, Wiley, & Gallagher, 1995). Stereotaxic surgeries were performed using predetermined coordinates (Baxter et al., 1995). Prior to surgery, rats were anesthetized with sodium pentobarbital (55 mg/kg ip; Butler Co., Dublin, OH) and

placed in a stereotaxic frame (Kopf Stereotaxic Instruments, Tujunga, CA), with the incisor bar set at 0.0 mm relative to the interaural line. The scalp was incised and reflected from the top of the skull, where four small holes were drilled to allow passage of the cannula; these craniotomies were located −0.75 mm posterior to bregma, ± 2.3 mm and ± 3.3 mm from midline. Rats in the NBM lesion group received bilateral infusions of 192 IgG-saporin (Chemicon, Temecula, CA) at a concentration of 4 μg/μl in Dulbecco's sterile saline solution (Sigma, St. Louis, MO). Using a microinjection unit (model 5000/5002, Kopf Stereotaxic Instruments, Tujunga, CA) mounted to the stereotaxic frame, a volume of 0.2 μl of the 192 IgG-saporin solution was infused via a 28-gauge syringe (Hamilton, Reno, NV) at a rate of 0.1 μl/min (i.e., 2 min per infusion) bilaterally into each hemisphere. For the medial sites, infusions were made at −7.8 mm below the surface of the skull, ± 2.3 mm from midline, and −0.75 mm from Bregma. For the lateral sites, infusions were made at −8.1 mm below the surface of the skull, ± 3.3 mm from midline, and −0.75 mm from Bregma. Once lowered, the cannula was left in place for 30 s to allow the brain to settle around the cannula prior to infusion. Following each infusion, the cannula was left in place for an additional 3 min to allow diffusion of the immunotoxin (or vehicle) away from the cannula tip. Surgical procedures were identical for rats in the sham-operated control group, with critical distinction that these animals received infusions of Dulbecco's sterile saline without 192 IgG-saporin. Following surgery, the incision was cleaned and sutured and rats were allowed two weeks for recovery prior to re-testing in the DNMTP task.

Histology. Upon completion of behavioral testing, rats received a lethal dose of sodium pentobarbital (80 mg/kg, i.p.; Sigma, St. Louis, MO) and received cardiac

perfusion with 0.9% saline solution for 5 min followed by 4% phosphate-buffered formalin solution for 30 min followed by 5% phosphate-buffered sucrose solution for 5 min. The perfusion solutions and the heads of the animals were kept on ice throughout perfusion to prevent degradation of the enzyme acetylcholinesterase (AChE). Perfused brains were extracted and placed in 10% phosphate-buffered sucrose solution for 24 hr before being transferred to a 25% phosphate-buffered sucrose solution for another 24 hours prior to freezing and sectioning (60 μm) using a cryostat.

Alternate sections were then stained for acetylcholinesterase (AChE) using the method of Baxter, Bucci, et al. (1995), or were stained for gamma-aminobutyric acid (GABA) using a monoclonal antibody to parvalbumin as described by Fitzpatrick and colleagues (1988); AChE-stained sections were examined to verify the placement and extent of the NBM lesions, whereas GABA-stained sections were examined to confirm sparing of GABAergic neural elements within the basal forebrain at the lesion sites.

Statistical Analyses. Pre-operative data was sorted to create the control group and the NBM lesion groups ultimately determined at Block 10 (final pre-operative block) based on pairs of animals matched for average performance and then randomly assigned to either the NBM lesion or sham lesion control groups as described above. First, analyses were conducted to provide evidence that the matched groups showed equivalent performance in acquiring the DNMTP task under both the no delay and the variable delay training conditions. Next, post-operative data (Block 11) were analyzed and compared to the immediately preceding pre-operative data (Block 10) in order to assess the effects of NBM lesions on DNMTP performance. The procedures for conducting these analyses are described below.

Data from the pre-operative, no delay training period was analyzed by mixed factorial (2 x 5) analysis of variance (ANOVA) with Group (NBM vs Control) as the between factor and Block (Blocks 1-5) as the within-group factor. Data from the pre-operative, variable delay training period were similarly analyzed by mixed factorial (2 x 3 x 5) ANOVA with Group (NBM vs Control) as the between factor, and with Delay (0, 60, and 120 s) and Block (Blocks 6-10) as within-group factors. Significant main within-group effects during the no delay or variable delay training periods were followed up with post hoc linear trend analyses in order to determine whether performance improved reliably across Blocks, and if so in which group.

Post-operative performance (i.e., Block 11) was compared to final pre-operative performance (i.e., Block 10) again using a mixed factorial (2 x 3 x 2) ANOVA with Group as the between factor (NBM vs. Control), and with Delay (0, 60, and 120 s) and Block (Blocks 10 and 11) as within-group factors. Additionally, to test the specific a priori hypothesis that post-operative performance in the NBM lesion group would be impaired only under the 60 and 120 s delay conditions (and not under the 0 s delay condition), two orthogonal contrasts on the Delay factor were conducted. Between- and within-group comparisons between performance under the 0 s delay condition and combined performance at the 60 and 120 s delays were conducted in order to determine whether NBM lesions caused a delay-dependent impairment in working memory; such delay-dependent impairment would be reflected by post-operative between-group differences only at the 60 and 120 s delays and not at the 0 s delay. A comparison between performance at the 60 and 120 s delays was also conducted to determine whether deficits at the 120 s delay exceeded those at the 60 s delay in the NBM lesion group.

Chapter 3

RESULTS

Pre-Operative, No Delay (0 s) Acquisition. Although groups were formed after

pre-operative training was completed (i.e., at Block 10), and prior to surgical

manipulations, pre-operative data are shown for the two groups that were subsequently

formed based on matching individual pairs of rats on the average percentage correct

across all delays on the final block of preoperative testing (see Methods). As shown in

Figure 1 (Blocks 1-5), both groups acquired the 0 s delayed alternation task equally well

(with performance in both groups reaching a level greater than 90% correct on Blocks 4

and 5). A mixed factor ANOVA (Group x Block) comparing percent correct in each

group on Blocks 1-5 confirmed these observations. Between-group comparisons were

not statistically significant, indicating that groups were adequately matched at the end of

pre-operative testing. A significant main within-group effect on the repeated factor of

Blocks yielded an $F_{(4, 32)} = 22.97$, $p < .001$. Post hoc tests confirmed that both groups

showed improved performance across testing Blocks 1-5; linear trend analysis yielded an

$F_{(1, 8)} = 65.92$, $p < .001$; no interaction occurred between trends associated with the two

groups, indicating that performance trends did not differ between groups. Consistent

with these post hoc analyses, the Group by Block interaction from the ANOVA was not

significant.

Pre-Operative, Variable Delay (0, 60, and 120 s) Acquisition. As shown in Figure 1 (Blocks 6-10), acquisition under the variable delay condition was also equivalent between groups; both groups showed a significant improvement in performance at all delays (i.e., 0, 60, and 120 s) across the five blocks of variable delay training. Additionally, both groups showed delay-dependent performance, with the highest level of performance occurring at the 0 s delay, intermediate levels of performance at the 60 s delay, and the poorest performance at the 120 s delay.

A mixed factor ANOVA (Group x Block x Delay) comparing percent correct in each group on trial blocks 6-10 confirm these observations. Performance in both groups varied as a function of the length of delay imposed, as revealed by a significant main effect for Delay, yielding an $F_{(2, 16)} = 75.05$, $p < .001$. A significant Delay by Block interaction ($F_{(8, 64)} = 2.34$, $p < .05$) demonstrates that the extent of across-block improvements in performance differed as a function of delay; across block improvements in performance were greater at the 60 and 120 s delays than at the 0 s delay for both groups, where both groups reached near ceiling levels (i.e., near 100% correct) at the 0 s delay. The absence of a significant Group by Delay interaction indicates that both groups responded equivalently at the various delays.

Post hoc linear trend analyses show that both groups showed across block improvement in performance during Blocks 6-10 at all delays (i.e., 0, 60, and 120 s). Linear trend analyses conducted on both groups at the 0, 60, and 120 s delays yielded $F_{(1, 8)} = 16.96$, $p < .01$, $F_{(1, 8)} = 22.98$, $p < .001$, and $F_{(1, 8)} = 10.99$, $p < .05$, respectively. No interaction occurred between group trends at any delay, indicating that performance trends did not differ between groups at either the 0, 60, or 120 s delay.
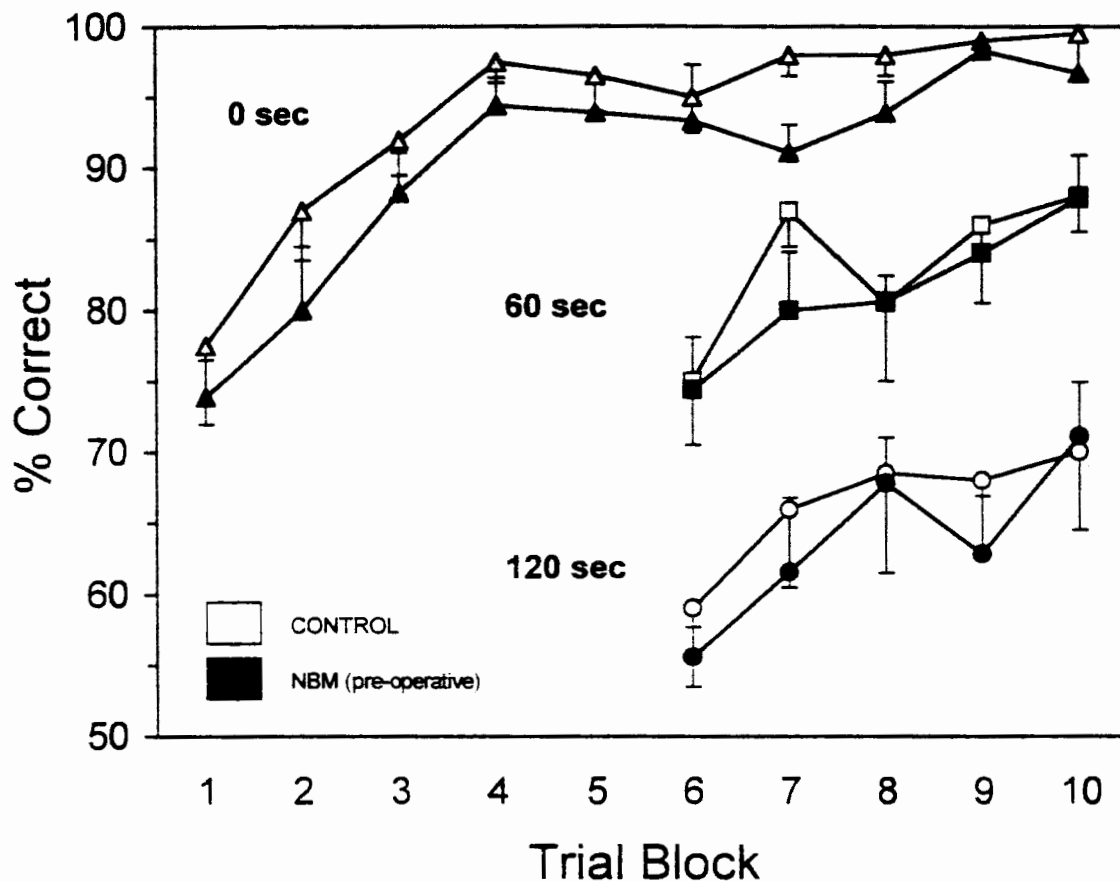
Figure 1. Pre-operative acquisition performance (mean ± SEM) of the delayed alternation Y-maze task in matched groups assigned to the NBM lesion (BLACK) or sham-operated control group (WHITE). During trial blocks 1-5, both groups showed significant improvements in performance at the 0 s delay (triangles; p < .001). During trial blocks 6-10, both groups similarly showed significant improvements at the 60 (squares) and 120 s (circles) delays and showed further improvement at the 0 s delay (triangles; p < .001). Delayed alternation performance in the matched NBM lesion and control groups did not differ at any delay.

Consistent with these post hoc analyses showing equivalent performance in the two groups, between-group comparisons from the ANOVA were not statistically significant. Similarly, neither the Group by Block, Group by Delay, nor Group by Block by Delay interactions from the ANOVA were significant, indicating that groups did not differ at any delay during variable delay training in Blocks 6-10. Overall, these data show that the matched groups performed equivalently in every respect during pre-operative training both in the no delay (i.e., Blocks 1-5) and the variable delay (i.e., Blocks 6-10) conditions. Additionally, these analyses demonstrate that performance varied as a function of delay in both groups, with poorer performance at longer delays.

Pre-Operative vs Post-Operative Delayed Alternation. Post-operative (Block 11) performance at the 60 and 120 s delays was impaired in the NBM lesion group as compared to the control group, while post-operative performance at the 0 s delay was equivalent in the two groups (see Figure 2). The magnitude of post-operative impairment at the 120 s delay, however, was not significantly greater than the 60 s delay in the NBM lesion group. Post-operative differences were not associated with differences in pre-operative performance (Block 10), which was equivalent between groups in all respects (reflecting the effectiveness of the pre-operative matching procedure). The specific analyses supporting these observations are described below.

The mixed factorial ANOVA comparing pre-operative performance (i.e., Block 10) to post-operative performance (i.e., Block 11) at each delay (i.e., 0, 60, and 120 s) in each group revealed a significant main effect for Group ($F_{(1, 8)} = 6.37$, $p < .036$) and Delay ($F_{(2, 16)} = 90.16$, $p < .001$), while the three-way interaction between Group, Delay,
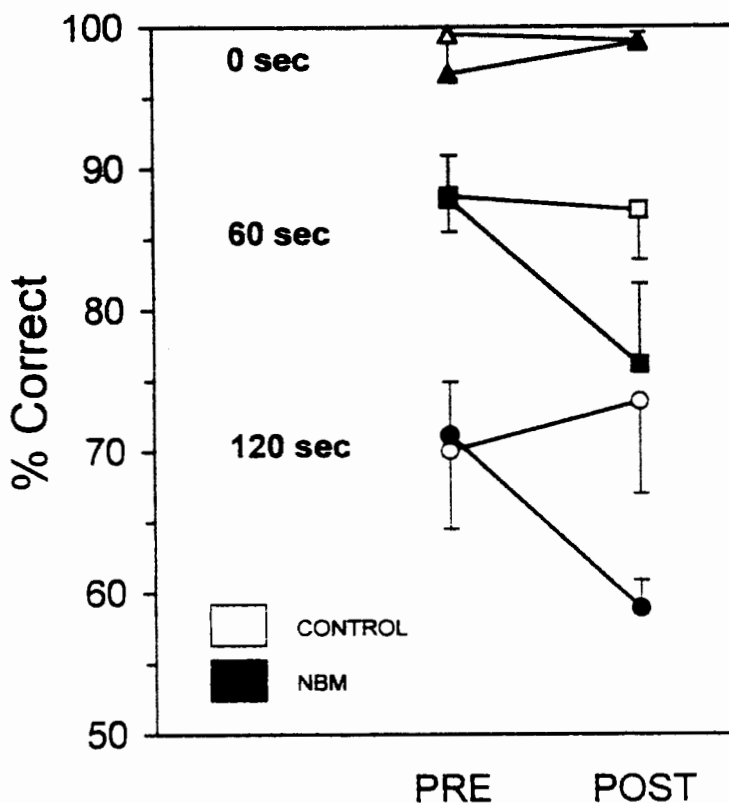
Figure 2. Delayed alternation performance (mean ± SEM) in the NBM lesion (BLACK) and sham-operated control (WHITE) groups on the final pre-operative trial block (PRE) and on the post-operative trial block (POST) at the 0 (triangles), 60 (squares), and 120 s (circles) delays. Performance at the 0 s delay did not differ between groups either before or after NBM lesions. In contrast, while pre-operative performance at the 60 and 120 s delays was equivalent in the two groups, post-operative performance at the 60 and 120 s delays was impaired in the NBM lesion group relative to control group performance ($p$ < .042). The magnitude of impairment at the 60 and 120 s delays in the NBM lesion group did not differ significantly.

and Block approached but did not reach statistical significance ($p < .086$). No other significant main effects or interactions occurred. The significant group effect was attributed to poorer overall performance in the NBM lesion group as compared to the control group. The significant delay effect demonstrates that both groups continued to perform more poorly at longer delays relative to performance at shorter delays. The absence of a significant Group by Delay by Block effect was anticipated by the prediction of no group difference at the 0 s delay; by comparing groups across delays, such equivalent performance at the 0 s delay would diminish apparent between-group differences. Consequently, the two planned a priori contrasts that were described previously were conducted (see Methods).

The first of these two a priori contrasts compared pre- (Block 10) and post-operative (Block 11) performance in each group under the 0 s delay condition to combined performance at the 60 and 120 s delays to test for potential delay-dependent impairments following NBM lesions. This contrast provided a significant three-way interaction between Group (NBM vs Control), Block (Block 10 vs 11), and Delay (0 s vs 60 and 120 s), yielding an $\underline{F}_{(1, 8)} = 5.83$, $\underline{p} < .042$. This contrast demonstrates that performance at the 0 s delay differed from combined performance at the 60 and 120 s delays in the NBM lesion and control groups as a function of test block (i.e., pre-operative vs post-operative). The effect is attributable to poorer post-operative performance at the 60 and 120 s delays (but not at the 0 s delay) in the NBM group as compared to controls.

The second a priori contrast compared performance in each group at the 60 and 120 s delays in order to determine if potential deficits at the 120 s delay exceeded those at
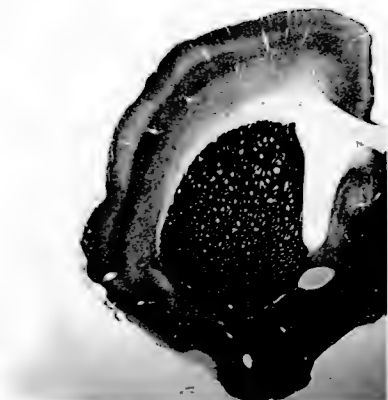
the 60 s delay in the NBM lesion group. These comparisons failed to yield statistical significance, indicating that the magnitude of NBM lesion induced impairment was not reliably different at the 60 and 120 s delay intervals.

Histology. As shown in Figure 3, the cortices of the NBM lesion rats showed profound absence of AChE-positive staining as compared to control brains, demonstrating the cortical cholinergic depletion resulting from SAP infusion into the NBM. Frontal, parietal, and posterior parietal cortices were consistently depleted of AChE in all NBM lesion group animals. In contrast, medial septal cholinergic innervation of hippocampus in the SAP NBM lesion group was found to be intact in all cases upon examination of posterior sections containing hippocampus, which showed dense AChE-positive staining (compare Figure 3E and 3F).

Consistent with the observation of deficient cortical AChE staining, brains from the NBM lesion group showed a loss of AChE-positive fibers and cell bodies in the region of the NBM (Figure 4B), whereas no such loss was observed in control brains (Figure 4A). Cell bodies of the diagonal band of Broca, as well as those of the medial septum, appeared intact in all cases in the NBM lesion groups, consistent with the intact AChE staining pattern observed in the hippocampus of these animals. Parvalbumin immunostaining of cells adjacent to the infusion sites was equivalent in NBM lesion (Figure 4D) and control (Figure 4C) brains, indicating that the SAP lesions did not damage GABAergic cell bodies or fibers of passage in the regions surrounding the NBM and were therefore specific to cholinergic neurons only.

Figure 3. Photographs of coronal hemi-sections stained for AChE taken at the level the

frontal, parietal, and posterior parietal cortex in a typical sham-lesion control rat (A, C,

and E) and a typical SAP NBM lesion rat (B, D, and F). The NBM lesion brain shows a

profound depletion of AChE throughout the cortex, whereas the control brain shows

normal dense staining and laminar distribution of AChE-positive fibers in neocortex.

Cholinergic projections from the MSA to the hippocampus remained intact following

NBM lesions, as evidenced by normal AChE staining in dorsal hippocampus (F).
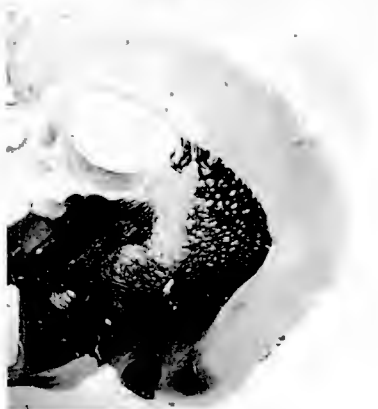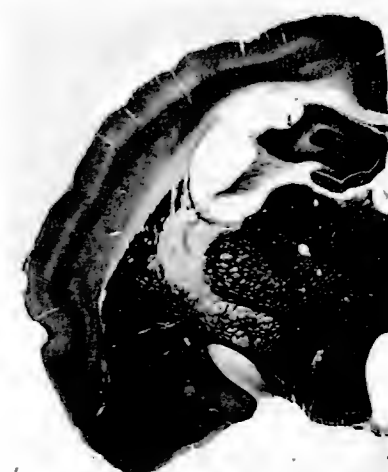
Figure 3

Figure 4. Photographs of sections taken at the level of the NBM, stained for AChE in a

typical sham-lesion control rat brain (A) and in a typical SAP NBM lesion rat brain (B),

and photographs of adjacent sections immunostained for parvalbumin in the same control

(C) and NBM lesion (D) brains. Note the presence of AChE-positive fibers and cell

bodies in the NBM of the control brain (A) and the absence of staining in the NBM lesion

brain (B). Parvalbumin immunostaining revealed parvalbumin-immunopositive

GABAergic neurons in both the control (C) and NBM lesion brain (D), demonstrating

that the SAP lesions did not affect GABAergic cells surrounding the NBM.

Figure 4

Chapter 4

DISCUSSION

The results of the current study demonstrate that a DNMTP task can be used to detect working memory impairments caused by selective lesions of the NBM. Following surgery, NBM lesioned rats were not impaired at the 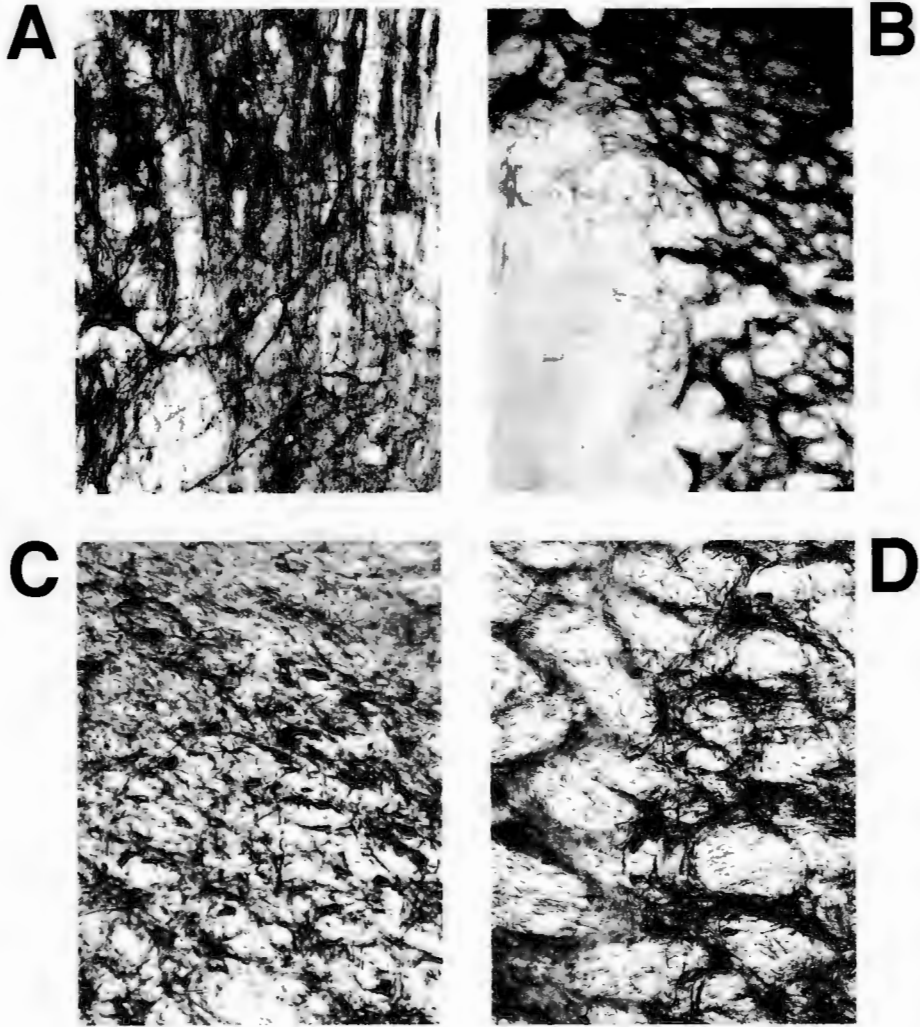0 s delay, which suggests that they remembered the rule for the DNMTP task (i.e., reference memory). In contrast, the NBM lesioned rats were impaired at the 60 and 120 s delays, suggesting a selective impairment in spatial working memory. The magnitude of impairment observed at the 120 s delay was marginally greater than the 60 s delay; however, the difference between performance on both delays was not statistically significant. A floor effect at the 120 s delay may have contributed to the lack of greater impairment detected at this delay; postoperative performance was just above chance levels at the 120 s delay.

Together, the AChE and parvalbumim-immunoreacted sections demonstrated that 192 IgG-saporin is indeed highly selective. Previous studies where working memory impairments were found that used less selective toxins are confounded because post-operative changes in behavior may have been due to neuronal damage in the NBM as well as other nearby non-cholinergic structures (Beninger et al., 1994; Misztal et al., 1996). The immunotoxin 192 IgG-saporin, however, produces selective lesions only of the cholinergic neurons in the NBM (Schliebs, Rossner, & Bigl, 1996; Walsh, Herzog,

Gandhi, Stackman, & Wiley, 1996). Thus, the working memory impairments observed here can reliably be attributed to damage of the cortically-projecting cholinergic neurons of the NBM.

Although others have explored possible impairments in working memory in animals with selective 192 IgG-saporin, the current results uniquely demonstrate a critical role for the NBM in supporting normal working memory. The inclusion of a 0 s delay and designing the study such that rats received extensive pre-training before lesioning separates our study from the earlier studies. The addition of a 0 s delay allows for the conclusion of intact reference memory (i.e., memory for the "rule"). Training rats before lesioning also ensured that the "rule" remained intact post-lesion. These data show that rats with cholinergic lesions using the immunotoxin 192 IgG-saporin have working memory impairments, but not reference memory impairments.

These results show that 192 IgG-saporin NBM lesions cause reliable impairments in working memory and thus may provide an important animal model for the working memory impairments found in Alzheimer's disease (e.g., Becker, 1988; Morris & Baddeley, 1988; Baddeley, Bressi, Della Sala, Logie, & Spinnler, 1991; Becker, Bajulaiye, & Smith, 1992; Belleville, Peretz, & Malenfant, 1996; Baddeley, Cocchini, Della Sala, Logie, & Spinnler, 1999). The temporal nature of the decay of performance in the DNMTP task (i.e., no impairment at 0 s delay but significant impairment at longer delays) suggests that the NBM serves a function in preventing the decay of memory over time (see also Hasselmo, 1995).

The intact reference memory for the pre-operatively acquired alternation rules in the current study is consistent with other studies. Butt and Hodge (1995) found that

ibotenic acid lesions of the NBM produced only negligible and brief disruption of

reference memory in an operant discrimination task. González, Miranda, Gutiérez,

Ormsby, & Bermúdez-Rattoni (2000) found impairments in both the conditioned taste

aversion and Morris water maze tasks when training followed NBM excitotoxic lesions.

However, when training preceded lesioning in these same tasks, no impairments in the

pre-operatively acquired prepositional rules were observed. Other studies also have

demonstrated that complex rule learning may be more susceptible to cholinergic lesions

of the NBM when training occurs post-operatively, whereas these same learned behaviors

are resistant to the effects of NBM lesions when acquired pre-operatively (e.g., Butt &

Bowman, in press; Butt, Noble, Rogers, & Rea, in press).

The basal forebrain cholinergic system in humans projects to the entire neocortex;

this system is comprised of the nucleus basalis of Meynert, the medial septum, and the

diagonal band of Broca (Perry & Hodges, 1999). In studies with excitotoxic lesions in

rats, a disruption of working memory despite intact reference memory have been found

(e.g., Hepler & Olton, et al., 1985; Hepler & Wenk, et al., 1985; Beninger & Jhamandas,

et al., 1986; Beninger & Wirsching, et al., 1986; Wozniak, Stewart, Finger, & Olney,

1989; Wozniak, Stewart, Finger, & Olney, et al., 1989; Biggan et al., 1991; Moran et al.,

1992; Beninger et al., 1994). Studies that used 192 IgG-saporin to lesion both the NBM

and medial septum have also found impairments in working memory with reference

memory (i.e., memory for the rule) intact (Steckler et al., 1995; Waite et al., 1995;

Leanza et al., 1996; Robinson et al., 1996; Dornan et al., 1997; McDonald et al., 1997;

Wrenn et al., 1999). One study has also found impaired working memory and intact

reference memory with selective 192 IgG-saporin lesions of the NBM alone (Dornan et

al., 1997). The combined information from these studies strongly suggests a critical role of the NBM cholinergic cortical projection system in supporting normal working memory function.

Pharmacological studies examining the contribution of GABA on working memory also support the results in the current study. DeSousa, Beninger, Jhamandas, & Boegman (1994) examined the role of GABAergic inputs to the NBM in spatial working memory. DeSousa et al. (1994) injected the GABA receptor agonists Baclofen and muscimol into the NBM. They tested rats in a double Y-maze spatial task in which working and reference memory were assessed. DeSousa et al. (1994) found that GABA agonists impair spatial working memory while leaving reference memory intact. Another study has also found results consistent with GABAergic disruption of working memory while sparing reference memory (Beninger et al., 1992). Consistent with the impairment due to the GABA agonists, GABA inverse agonists injected in the intra-NBM have been shown to augment working memory function (Smith, Beninger, Mallet, Jhamandas, & Boegman, 1994; Mason, Mallet, Jhamandas, Boegman, & Beninger, 1999). These studies of GABAergic modulation of cholinergic function in the NBM provides further support that the NBM is involved in working and not reference memory.

Neurochemical studies of the effects of the corticocortical and septo-hippocampal cholinergic pathways on attentional tasks also supports the results found in the current study. Durkin (1994) trained mice in a delayed (5 or 6 min) non-match to sample task in an 8-arm radial maze and measured the sodium-dependent high-affinity choline uptake. Durkin found that the NBM cortical pathway was active during both the 5 and 60 min retention intervals. On the contrary, the septo-hippocampal pathway was active only

during the first several minutes during both retention intervals. Therefore, working memory demands corresponded to changes in the NBM cortical pathway, but not septo-hippocampal pathway. Durkin determined that the results suggested that the NBM and not the septo-hippocampal pathway is critically involved in the maintenance of working memory. The results of the current study indeed support Durkin's interpretations.

Other research that used 192 IgG-saporin infusions to make combined NBM and MSA lesions have demonstrated more severe working memory impairments in rats with combined lesions compared to rats with either NBM or MSA lesions alone (e.g., Steckler et al., 1995; Waite et al., 1995; Leanza et al., 1996; Robinson et al., 1996; Dornan et al., 1997; McDonald et al., 1997; Wrenn et al., 1999). These studies are informed by date from the current study, which shows that the NBM makes a substantial contribution to working memory function.

These data support a role for the specific involvement of the NBM cholinergic cortical projection system in working memory. This is consistent with working memory research in which working memory impairments associated with NBM cholinergic cell loss were found (Baddely, Bressi, Sala, Logie, & Spinnler, 1991; Belleville, Peretz, & Malenfant, 1996). According to Albert (1996), the NBM, which has projections to the neocortex, also receives many projections from subcortical structures. Consequently, the NBM can be viewed as a critical element in a circuit flowing from subcortex to cortex. Disruption of this pathway by NBM lesions effectively disrupts communication between subcortical and cortical areas. This disruption between subcortical and cortical areas may affect attentional tasks such as the DNMTP task, because such a task requires rapid and

simultaneous integration and processing of many different types of information (i.e., spatial location, alternation rule, sustained attention, set-shifting, & self-monitoring).

More research needs to be conducted in order to determine if AChE inhibitors will compensate for cholinergic cell loss and thus alleviate working memory impairments such as those found in the current study. Tacrine, an AChE inhibitor is used to treat symptoms of Alzheimer's dementia, has been found to modestly benefit patients with relatively mild to moderate dementia (Reiman & Caselli, 1999). These data are consistent with the modest therapeutic effects observed with AChE inhibitors in working memory of patients with Alzheimer's disease. The relative contribution of non-cholinergic NBM cells to working memory remains to be determined. However, these data support a contribution of cholinergic NBM cells to working memory.

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