The Effect of Selective Cholinergic Lesion of Medial Septum on Recognition Memory

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THE EFFECT OF SELECTIVE CHOLINERGIC LESION OF
MEDIAL SEPTUM ON RECOGNITION MEMORY

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ABSTRACT

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The objective of this study was to clarify the role of septal-hippocampal cholinergic neurons in object and spatial recognition memory. Stereotaxic-surgical infusion of the selective cholinergic neurotoxin, 192IgG-saporin (SAP), into the medial septum (MS) of Sprague-Dawley rats was utilized to establish an animal model of cholinergic deficit of the septal-hippocampal tract, which had been expected as a pathologic model of memory impairment for Alzheimer’s disease (AD). Three types of recognition memory were examined: retrograde object recognition, anterograde object recognition, and anterograde spatial recognition. These were examined with a modified version of a standard object recognition paradigm. For retrograde memory retention testing, rats received SAP after training; in contrast, for anterograde retention testing, rats received SAP before training. The time that the rats spent exploring familiar and novel objects or familiar objects in a novel location was measured. The effects of SAP on the three types of recognition memory were tested and compared to control animals. There was no significant difference in the mean exploration ratios (MERs) between control rats infused with artificial cerebrospinal fluid (aCSF) and control rats that did not receive...
surgery (NOR). The MERs for both control groups were in the range between 0.6-0.7, consistent with the object recognition testing literature. These results indicate that the infusion surgery itself had no effect on object or spatial recognition memory and that the methodology for object and spatial recognition developed for this study worked well. SAP lesioned rats did not demonstrate impairment of retrograde object recognition memory (0.68±0.04 vs 0.67±0.03, \( p = 0.888 \)) and also displayed normal anterograde object recognition memory (0.67±0.04 vs 0.66±0.02, \( p = 0.866 \)) compared to control rats. However, compared to controls, SAP rats were significantly impaired in anterograde spatial recognition memory as reflected in the fall of the MER for the SAP group to chance levels (0.51±0.04 vs 0.62±0.02, \( p = 0.0081 \)). These findings suggest that the septal-hippocampal cholinergic neurons play an important role for spatial recognition memory, but not for object recognition memory and indicate that the septal-hippocampal cholinergic deficit may be responsible for the mild memory impairments shown in the early phase of AD. This study first provides the comparable data of selective cholinergic lesion of septal-hippocampal tract in object recognition memory and spatial recognition memory and provides additional support for the theory that the hippocampus is critical for recognition memory that utilizes associations or recollection, but not for familiarity.
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## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>iv</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>vi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>ix</td>
</tr>
<tr>
<td>List of Abbreviation</td>
<td>x</td>
</tr>
<tr>
<td><strong>I. Introduction</strong></td>
<td>1</td>
</tr>
<tr>
<td>A. Statement of Problem</td>
<td>1</td>
</tr>
<tr>
<td>B. Literature Review</td>
<td>2</td>
</tr>
<tr>
<td>1. Memory</td>
<td>2</td>
</tr>
<tr>
<td>2. Amnesia</td>
<td>11</td>
</tr>
<tr>
<td>3. Anatomy of the septo-hippocampal cholinergic system</td>
<td>13</td>
</tr>
<tr>
<td>4. Mnemonic studies of cholinergic lesion induced by 192IgG-saporin</td>
<td>23</td>
</tr>
<tr>
<td>5. Task used to assess recognition memory</td>
<td>27</td>
</tr>
<tr>
<td>C. Hypothesis and Aims</td>
<td>30</td>
</tr>
<tr>
<td><strong>II. Experimentals</strong></td>
<td></td>
</tr>
<tr>
<td>A. Materials and equipments</td>
<td>31</td>
</tr>
<tr>
<td>1. Facilities</td>
<td>31</td>
</tr>
<tr>
<td>2. Animals</td>
<td>31</td>
</tr>
<tr>
<td>3. Chemicals and Drugs</td>
<td>31</td>
</tr>
<tr>
<td>4. Materials</td>
<td>32</td>
</tr>
<tr>
<td>5. Equipment</td>
<td>33</td>
</tr>
<tr>
<td>6. Computer Software</td>
<td>33</td>
</tr>
</tbody>
</table>
B. Methodology and Procedures .................................................................35
   1. Animal Conditions ...........................................................................35
   2. Animal Surgery ..............................................................................35
   3. Novel object recognition task ..........................................................36
   4. Animal behavioral studies ...............................................................42
   5. ChAT (Choline acetyltransferase) Assay ..........................................42
   6. Statistical Analysis .........................................................................43

III. Results

A. ChAT Assay
   Effects of SAP infusion in the MS on ChAT activity in frontal cortex and
   hippocampal formation. ........................................................................44

B. Animal behavioral studies .................................................................
   Part 2: The effect of SAP treatment on retrograde recognition memory ....47
   Part 3: The effect of SAP treatment on anterograde recognition memory .48

IV. Discussion ..........................................................................................53

V. Conclusion ............................................................................................62

VI. References ..........................................................................................63
LIST OF FIGURES

Figure 1: Overview of memory processes and memory systems ...........................................6

Figure 2: Dissected human hippocampus next to a specimen of hippocampus leria (seahorse) ...............................................................................................................15

Figure 3: The hippocampal formation consisting of three distinctive divisions: hippocampus (proper), dentate gyrus and subiculum. ........................................16

Figure 4: The internal organization of hippocampal formation........................................ 17

Figure 5: Direction of information flow within the hippocampal formation............... 18

Figure 6: The distribution of the cholinergic neurons and their projections in the central nervous system ...................................................................................................... 20

Figure 7: Medial septum and diagonal band of broca (coronal section)........................... 21

Figure 8: Fimbria /fornix bundles ..................................................................................... 22

Figure 9: Apparatus for Novel Object Recognition Task ................................................. 34

Figure 10: The Floors of the Open Field ........................................................................... 38

Figure 11: Timeline for behavioral experimental procedures........................................... 39

Figure 12: Procedures for recognition memory testing ..................................................... 41

Figure 13: Effect of SAP infusion in the MS on the ChAT activity of hippocampus and the frontal cortex for the lesioned rats ................................................................. 45

Figure 14: The effect of surgery procedures on recognition memory ............................... 50

Figure 15: Effects of SAP treatment on retrograde object recognition memory ............ 51

Figure 16: Effects of SAP treatment on anterograde object recognition memory ......... 51

Figure 17: Effects of SAP treatment on anterograde spatial recognition memory .......... 52
LIST OF ABBREVIATIONS

ACh - Acetylcholine
ACHE - Acetylcholinesterase
AD - Alzheimer s disease
aCSF – Artificial Cerebrospinal fluid
CBF - Cholinergic basal forebrain
ChAT - Choline acetyltransferase
CNS - Central Nervous System
CON – Control
DBB - Diagonal band of Broca
FX - Frontal Cortex
HIPP - Hippocampus
hDB-horizontal limb of diagonal band
i.c.v. – intracerebroventricular
LDT – lateraldorsal pontine tegmental
MS - Medial septum
MER - mean exploration ratio
MTET – mean total exploration time
NMDA - N-methyl-D-aspartic acid
NOR-Normal
NBM/BSA - Nucleus Basalis of Meynert
PPT – pedunculopontine tegmental
p75NGR - Low affinity nerve growth receptors
SI - substantia innominata
SAP - 192IgG-Saporin
sec – seconds
vDB-vertical limb of diagonal band
I. INTRODUCTION

A. Statement of Problem

Cholinergic neurons of the medial septum (MS) have long been recognized for playing an important role in learning and memory. This was in part due to the anatomic relationship between the MS and the hippocampus, a structure known to be important for memory function. However, discrepant results from recent research that selectively destroyed cholinergic innervations from the MS to the hippocampus suggested that the role of septal-hippocampal acetylcholine may have been overestimated (1), and that lesion of the septal-hippocampal cholinergic neurons may not be an adequate model for memory impairments of Alzheimer’s disease (AD) (2). On the one hand, the discrepant results could be related to a number of factors such as the site and degree of lesion, the memory demands or the complexity of the specific protocols used in different laboratories, or the cognitive processes involved in different memory behavioral tasks. On the other hand, behavioral impairments induced by intracerebroventricular (i.c.v) administration of a selective cholinergic neuron toxin might indicate that cholinergic neurons outside of the septal-hippocampal tract could also be involved in memory function. The goal of the present study was to elucidate the role of cholinergic neurons of the medial septum in recognition memory, a basic memory function that underlies many learning and memory tasks, via using a novel object recognition paradigm, a simple learning and memory task. Thus the results of this study will help to clarify the discrepant results of the studies with the lesion of septal-hippocampal cholinergic neurons tested in more complicated learning
and memory tasks and appropriately define the role of this pathologic model in the studies of memory impairments involved in AD.

B. Literature Review

1. Memory

1.1 What is memory?
Memory refers to the storage, retention and recall of information including past experiences, knowledge, and thoughts, or as one of prominent theory conceives of memory, a flow of information through multiple processes and stages (3). The duration and intensity of memories vary depending on the content of the information. For example, information related to personal experiences (episodic memory) is more easily remembered than information obtained from textbooks (semantic memory). Memories for novel or exciting information tend to be stronger than ordinary or uninteresting information. The precise biological processes involved in memory are not fully understood, but memories are believed to be a set of encoded neural connections that result from changes in the number of connections or connection strengths between neurons of the central nervous system (4).

1.2 Relationship of memory to learning
According to Eric Kandel (2000), memory is the process by which knowledge of the world is encoded, stored, and later retrieved; learning is the process by which the knowledge of the world is acquired (5). By these definitions, memory is nothing but a fundamental mental process for learning. Learning and memory are closely interrelated: memory underlies learning and learning improves memory, but they are distinct.
Learning is not merely a matter of memory but includes other cognitive factors such as motivation, comprehension and reasoning. Compared to learning, memory function is more constant and is a fundamental mental process associated with normal brain function, whereas, learning is more variable and is a result of individual’s experience. For example, in terms of memory, people share common features (e.g. easy recall of episodic memories, more effort needed for semantic memories), however, with learning, there is increased individual variability (e.g. the grades of an examination for a typical class usually show substantial variability in performance). Even though a memory deficit can lead to a learning deficit, a learning deficit is not necessarily related to a memory deficit, e.g. dyslexia. A learning deficit can be easily distinguished from memory deficit in human beings. However, it is not easy in animal studies. Therefore, one must exercise caution when designing behavioral tasks/tests of learning and memory, otherwise we may erroneously categorize a deficit in learning as memory impairment or overlook a memory deficit due to the use of an insensitive behavioral task/test.

1.3 Memory processes

Memory is more than a single process. Three or four distinct processes are believed to be involved in memory function: encoding, consolidation/storage and retrieval (in older models consolidation and storage are usually considered as one process but in newer models they are often considered as separate processes) (3, 6).

- Encoding is a process of converting sensory information into a form that can be held for a limited duration (formation of short-term memory)
- Consolidation involves the conversion of encoded information into a form that can
be retained for indefinite periods of time (formation of long-term memories)

- Storage is the structural and functional deposition of memories into brain areas (maintenance of the long term memories)
- Retrieval is the process whereby a stored memory is returned to consciousness (working memory)

1.4 Memory system

1.4.1 Definition of memory systems

Concepts of memory are often confused between the forms/types of memory and memory systems. These concepts are not equivalent (7). The criteria for designating a type of memory are not stringent. For example, one can think of verbal memory, recognition memory, and olfactory memory as different kinds of memory. A distinction of this sort can help to describe and organize empirical facts but is purely descriptive and does not necessarily define a memory system. The criteria for memory systems are more stringent. A memory system is defined in terms of mechanisms and principles of operational processes (8). Memory systems are distinguished in terms of different behavioral and cognitive functions, different neural structures and neural mechanisms, different operations, differences in the timing of memory appearance, and differences in the format of represented information (7). An intact memory system enables one to perform a large number of tasks of a particular class or category, regardless of the specific information content of the task.
1.4.2 Classification of memory systems

Three categories of memory systems have been widely accepted: sensory memory, working memory, and reference memory that correspond to the three stages in the flow of memory processing (3, 9, 10; figure 1).

- Sensory memory is also referred to as immediate memory or sensory register since the function of sensory memory is mainly to process immediate sensations. Sensory memory has a large capacity for unprocessed information but is only able to hold accurate images of sensory information momentarily. Without attention, sensory memories usually last about one second. With attention, sensory memory is normally processed into working memory.

- Working memory is often referred to as short-term memory since the information in working memory is of relatively short duration unless it is consolidated into reference memory. Working memory is not merely a temporary holding function, but also involves the manipulation of incoming information (11, 12, 13). Working memory is of limited capacity and susceptible to interruption. The greater the information load, the shorter the duration of working memory. Rehearsal within working memory (repetition of new information) improves the retention of working memory but not reference memory.

- Reference memory, also referred to as long-term memory, is a system in which memories are stored for an indefinite period of time. “Long” in this sense means something between a few minutes and several years or even lifelong. Long term memory is the ultimate stage of memory processing. Compared with working memory, the information stored in reference memory is relatively stable, permanent.
and insensitive to interruption or disruption. In contrast to working memory, reference memory has unlimited capacity and is maintained in an unconscious state. The retrieval of information from reference memory to working memory is a type of rehearsal that facilitates retention of reference memory (an aphorism says: “use it or lose it”).

Figure 1. Overview of memory processes and memory systems

1.5 General classification of memory

The general classification of memory (descriptive forms of memory) varies in terms of duration, nature/content, and retrieval of information.

1.5.1 Classification based on duration of information

Memory can be categorized according to duration. Temporal categories of memory consist of four classes based on the length of time that the memory lasts (10).

- Immediate memory/sensory memory (within seconds)
- Short-term memory (seconds to minutes)
- Intermediate-term memory (minutes to hours)
- Long-term memory (days to years)
Simply considering working memory as short term memory and reference memory as long term memory is inappropriate since overlap exists between working memory and reference memory in terms of duration. The boundary/delimitation between the working memory and reference memory should not be the duration of memory but rather the process of consolidation.

1.5.2 Distinctions between working memory and short term memory

Working memory and short term memory are often used synonymously. However, the two terms are not identical (10, 12, 13). Unfortunately, the mixed-use of short-term memory and working memory is still common in the literature. A result of the confused terminology can be misunderstanding in terms of the design and interpretation of study results. Recently, a study by Shrager and colleagues (2008) suggested that traditional tests for working memory such as a short time delay between training and testing were inaccurate since reference memories might also be formed even within the short interval of the time delay (14). In their study, rather than using time as a criterion to discriminate between working memory and reference memory, they used the vulnerability of memories to distraction. Tasks that depended on working memory were sensitive to distractions, whereas tasks that relied on reference memory were not. The results of the investigation indicated that the transition of information between working memory and reference memory could occur very early (the processing of working memory and reference memory may be concurrent) and that the medial temporal lobe was involved in reference memory, but not in working memory.
Working memory is a limited-capacity storage system involved in the maintenance and manipulation of information over short periods of time (15). It consists of three components: a phonological loop, a visuospatial sketch pad, and a central executive.

- The phonological loop is responsible for auditory and verbal information such as phone numbers, the names of people, or a general understanding of what other people are talking about. It is a system specialized for language.
- The visuospatial sketch pad is responsible for visual and spatial information including information about the position and properties of objects.
- The central executive co-ordinates the activity of both the phonological loop and the visuospatial sketch pad. Imagine the following scenario: you are driving a car and your friend in the passenger seat gives you directions. The directions are given verbally which are handled by the phonological loop while the perceptions of the traffic, street lights, etc. are processed by the visuospatial sketch pad. If you attempt to follow the directions given by your friend it is necessary to combine both kinds of information: the verbal and the visual information. The central executive mediates the connection between the two components of working memory. The roles of the central executive include encoding of information; control of conscious attention, control of flow of information (link the sensory memory to the reference memory) and initiating movements.

Short-term memory has been defined as a subtype of working memory (12). However, in another definition, working memory was referred to as a special sort of short-term memory (10). In other literature, short-term memory is often considered the same as
working memory. As we can see, there is an overlap in definitions of these two concepts. Working memory is a dynamic process involving the temporary retention and transformation of information in support of cognitive activity, whereas short term memory describes a more or less passive temporary memory store. It may be proper to say that short-term memory reflects only one feature of working memory.

1.5.3 Classification of memory based on the nature and content of information

Memory can also be divided into two categories according to the nature of the memory: declarative memory and non-declarative memory. Declarative memory is a kind of memory that provides the basis for the conscious recollection of facts and events and refers to memory for words, scenes, faces, and stories. It is assessed by conventional tests of recall and recognition (16). “Declarative” signifies that the memory can be brought to mind and that its contents can be declared. Non-declarative memory is a collection of non-conscious memories related to skills and habits, simple forms of conditioning, the phenomenon of priming and other functions in which experiences can change how we interact with the world (17). Non-declarative memories usually occur as modifications within specialized performance systems and are expressed through performance rather than recollection.

Declarative memories can be further divided into two subcategories according to the contents of the memory: episodic memory and semantic memory. Episodic memory is related to personal experiences and usually includes contextual information such as time
and location. Semantic memory is comprised of factual knowledge and seldom includes contextual information related to when and where the memory was acquired.

1.6 Classification of retrieval/recall

Recall is the process of retrieving information from unconscious reference memory into conscious working memory. Recall often depends on a retrieval cue, a hint or prompt that triggers the memory. The tip-of-the-tongue phenomenon occurs when one becomes conscious of difficulty recalling information from reference memory. This phenomenon suggests that recall is not a simple all-or-none process. Based on the involvement of the retrieval cues, recall can be divided into three types: free recall, cued recall, and recognition. Free recall is the retrieval of information with no cues. Cued recall is the retrieval of information in response to a cue stimulus or limited cues. Recognition is just the identification of a stimulus that has been exposed before. As we all know, recognition is usually the easiest test of recall. Recognition memory is a type of memory tested via recognition.

1.7 Recognition memory

Recognition memory refers to the capacity to judge recently encountered stimuli as familiar and is one of the most widely studied examples of declarative memory (17). Recognition memory is a fundamental facet of our ability to remember. It forms an integral component of declarative memory which is often impaired or lost in patients with amnesia (18). In humans, bilateral damage restricted to the hippocampal region impaired performance in standard tasks of recognition memory (19). Similar findings were also
observed in nonhuman primates (20) and rodents (21). Recognition memory is widely viewed as consisting of two component processes: familiarity and recollection (18, 22, 23). Familiarity, also referred as to a “know” response, involves a conscious sense of simply knowing that a particular stimulus had been previously presented. This sense of familiarity usually occurs immediately upon re-exposure to the item. Recollection, frequently referred to as a “remember response” that usually occurs more slowly than the “know response”, and involves remembering specific contextual details about a prior learning episode. In one study, AD patients were significantly impaired in “remember” responses but spared in “know” responses compared to the normal subjects (70).

2. Amnesia

Amnesia is an abnormal lack of or loss of memory, especially the loss of episodic memory, that cannot be attributed to the normal process of forgetting (24). Amnesia includes the loss of reference memories (storage failure with an emphasis on state) or the inability to acquire new memories (impairments in consolidation with an emphasis on action; 25). Almost anything that can cause brain damage can bring about a loss of memory including impaired blood supply (e.g. stroke), infections (e.g. meningitis), neurodegenerative diseases (such as AD) or trauma (car accidents). Damage to the hippocampus and adjacent structures is associated with amnesia in particular.

Amnesia is a primary symptom of Alzheimer’s disease. In early AD, patients behave in a way that resembles age-related-forgetfulness. As the disease progresses, individuals frequently cannot remember events that occurred only several minutes ago. AD is most
commonly diagnosed when patients develop disorientation to time, space and location (26). As an example, my aunt, who died in 2006, was not diagnosed with AD until the day she became lost in the neighborhood where she had lived for more than ten years. As AD becomes advanced, the information that has long been stored in reference memory is also lost. In the late stages of AD, many patients fail to recall their own history and even lose the ability to recognize family members and friends.

**Types of amnesia**

There are two typical types of amnesia: anterograde amnesia which is memory loss for events that occur following brain damage; and retrograde amnesia, memory loss for events that occurred prior to brain trauma.

**An example of amnesia: Patient H.M.**

Patient H.M. is one of the best known cases of a patient with amnesia. The case has been widely studied since the 1950s and has contributed substantially to the development of contemporary theories of memory and cognitive neurophysiology. H.M., who is still living today, is the subject of an ongoing investigation of memory function in a long term care institute located in Hartford, Connecticut (27). In 1953 at the age of 27, in order to relieve the suffering from an intractable seizure disorder, H.M. underwent a surgical resection of the bilateral hippocampus and adjacent brain area. The surgery succeeded in reducing the severity of the seizures; however, it also produced an unexpected anterograde and retrograde amnesia. “Forgetting the events of daily life as quickly as they occurred” was the comment of his doctor, W.B. Scoville in his report in 1957 (28). As an example, if someone were to walk into his room, introduce themselves and then leave, H.M. would not be able to recognize the person when they returned five minutes later.
Furthermore, many years after his surgery, H.M. does not know his age or the current date. He does not know where he is living and the status of his parents. H.M. does not remember the events that occurred the week prior to his surgery and has only a blurred memory of his personal history since high school, many years prior to the surgery. In short, H.M. has a severe impairment in the ability to form any new long-term memories and a moderate loss of long-term memories acquired before the surgery. However, his remote long term memory and working memory are spared. “He can remember childhood experiences and the knowledge of the world he acquired early in his life. H.M. can keep track of a conversation, his train of thought, and is capable of memory consolidation, especially for emotional memories (31).

3. Anatomy of the Septal-hippocampal Cholinergic System

The septal-hippocampal cholinergic system consists of the hippocampus, the medial septum and vertical limb of diagonal band of Broca (MSvDB), and the interconnections between them (32). The hippocampus and the MSvDB are connected mainly via fimbria/fornix bundle fibers.

3.1. Hippocampus

The hippocampus is a part of the limbic system, located within the temporal lobe of each cerebral hemisphere and is a major part of the medial temporal lobe.

3.1.1 Nomenclature of the hippocampus and adjacent structures
Although the hippocampus has long been recognized as a key structure involved in memory formation and spatial navigation, there is a lack of consensus for the terms that describe the hippocampus and the adjacent cerebral cortex. Three terms often found in the literature include “hippocampus”, “hippocampal formation”, and “hippocampal region”. Sometimes these terms are used synonymously; sometimes are not, e.g. the hippocampus is also referred to as the hippocampus proper (34). In short, the nomenclature of the hippocampal formation is variable and exactly which components are included may differ depending on the source. For example, in some literature, the adjacent structures such as entorhinal cortex (24, 35), perihinal, and postrhinal/parahippocampal cortices (36) are also considered components of hippocampal formation or hippocampal region. As a result, variations of nomenclature/definitions increase the difficulty of properly interpreting the results of studies related to the hippocampus and surrounding regions. Finally, a more comprehensive term “medial temporal lobe” is used to describe the hippocampus and its adjacent structures. The medial temporal lobe consists of two parts: the hippocampal region and the parahippocampal region. The hippocampal region includes the hippocampal formation consisting of the hippocampus proper, dentate gyrus and subiculum. The parahippocampal region consists of entorhinal, perirhinal and postrhinal/parahippocampal cortices (37).

3.1.2 Neuroanatomy of hippocampus (33–42)

The term hippocampus is derived from the Greek meaning seahorse. Actually the seahorse-like structure consists of the hippocampal formation and its efferent pathway, the fimbria/fornix bundles (Figure 2). The hippocampal formation is the “ancient”
(archicortex) part of the cerebral cortex composed of three principal cell layers rolled up inside the temporal lobe of the brain. It comprises three distinctive divisions in terms of morphology and connection: the hippocampus (proper), the dentate gyrus, and subiculum. These structures are roughly organized as strips running rostrocaudally within the temporal lobe (Figure 3).

Figure 2. Dissected human hippocampus next to a specimen of *hippocampus eria* (seahorse) (Touretzksy, D.S., 2007)
Figure 3. The hippocampal formation consisting of three distinctive divisions: hippocampus (proper), dentate gyrus and subiculum. (Kandel, ER, JH Schwartz and TM Jessell, 2000)
(1) The internal organization of hippocampal formation

The hippocampus proper in coronal section is a C-shaped structure with a characteristic laminar organization, consisting primarily of pyramidal cells and associated internurones. In rats the hippocampus proper is subdivided into three regions: CA1, CA2, CA3, but in humans, four regions: CA1, CA2, CA3 and CA4. The letters CA come from the Latin words *cornu ammonis*, which means “Ammon’s horn” in English. Like the hippocampus proper, the dentate gyrus is also a C-shaped laminar structure but smaller, that is interlocked with “big C” of the hippocampus proper and together they form an S-shaped structure. It is noteworthy that the cells found in dentate gyrus are not pyramidal cells but granule cells which play an important part in neurogenesis. The subiculum appears to be an extension of the hippocampus proper but actually is part of the proximal parahippocampal gyrus that connects the distal parahippocampal gyri with the hippocampus proper. That may be the reason that the subiculum is not considered a part of the hippocampal formation in some texts (38) (figure 4).

Figure 4. The internal organization of hippocampal formation (Touretzksy, D.S., 2007)
(2) Unidirection of information flow within the hippocampal formation

The information that flows through the hippocampal formation is mainly unidirectional. Pyramidal cells of the entorhinal cortex project their axons to the dentate gyrus to synapse on granule cells. Granule cell axons, termed mossy fibers, synapse on pyramidal cells of the CA3 region which in turn send their axons, termed Schaefer collaterals, to pyramidal cells of CA1. These Schaefer collateral axons also form the fimbria/fornix bundles through branches. Finally, the pyramidal cells of CA1 project their axons back to the entorhinal cortex either directly or via the subiculum, thereby forming a loop (figure 5).

Figure 5. Unidirection of information flow within the hippocampal formation(Touretzksy, D.S., 2007)
(3) Afferent and efferent pathways

The major afferents to the hippocampal formation are from the entorhinal cortex and, to a lesser extent, the septal area. There are also other inputs including those from the contralateral hippocampus, hypothalamus, amygdala, thalamus, locus ceruleus (noradrenergic projections), raphe nuclei (serotonergic projections), and ventral tegmental area (dopaminergic projections). The entorhinal area serves as an important gateway between the cerebral cortex and the hippocampus. Compared with input from the entorhinal cortex, the septal input is modest (33). Fibers from the septal nuclei reach the hippocampus via the fornix, the major efferent tract from the hippocampus and subiculum and contains about 1,200,000 fibers (38). The fornix projects to the mamillary nuclei, septal nuclei, and anterior nuclei of the thalamus. Another major efferent pathway from the hippocampus and subiculum is via the entorhinal cortex that projects to limbic, sensory-specific, and multimodal association cortical areas. In short, the hippocampus is well connected to the other brain areas via the entorhinal cortex, fornix and other pathways.

3.2. Medial septum/Diagonal band of Broca

3.2.1 Basal forebrain cholinergic system

The basal forebrain is a group of large neurons that are located ventral and rostral within the brain inferior to the striatum. They provide primary cholinergic innervations of the cerebral cortex, including the nucleus basalis (of Meynert), diagonal band, medial septum and substantia innominata (figure 6). Basal forebrain cholinergic neurons can be
classified into three groups (figure 6): the medial septal group (medial septal nucleus and vertical limb of the diagonal band, MSvDB) that project cholinergic axons mainly to the hippocampal formation, the lateral and ventral septal groups (the horizontal limb of diagonal band, hDB and substantia innominata, SI) that project to the olfactory bulb and the parahippocampal region such as entorhinal cortex (39), and the nucleus basalis group (nucleus basalis of Meybert, BAS/NBM) that project widely to frontal, parietal, and temporal neocortices and amygdala (40; 41). The degeneration of these cholinergic neurons of the basal forebrain is the earliest and most consistent pathological change seen in AD (42).

Figure 6. The distribution of the cholinergic neurons and their projections in the rodent central nervous system (Everitt, B.J. and Robbins, T.W., 1997)
3.2.2. Cholinergic innervation to the hippocampal formation—medial septum and vertical limb of diagonal band of Broca (MSvDB)

Even though the medial septum and the diagonal band of Broca are often classified as separate nuclei, they are actually continuous and no anatomical boundary exists between them (figure 7). The diagonal band of Broca can be further divided into two parts: the horizontal limb of diagonal band of Broca (hDB) and the vertical of diagonal band of Broca (vDB). As mentioned above, the medial septal nuclei provide the major cholinergic efferents to the hippocampus. Thus MSvDB is often referred as one functional unit. Apart from the projections to the hippocampal formation, the MSvDB also projects to the cingulate cortex, midbrain and hebenula nuclei in a less extensive manner (34, 41). Besides cholinergic neurons (40-50%), the MSvDB also has a minor cell group of GABAergic neurons (10-20%) that may also play a role in the function of MSvDB.

Figure 7. Medial septum (MS) and diagonal band of Broca (vDB, hDB) in rodent CNS (coronal section) (Sami ikonen, 2001)
3.3 Fimbria/fornix bundles

The hippocampus and the MSvDB are connected mainly by the fimbria/fornix bundles, the second afferent and efferent pathways of the hippocampus (the first is the angular bundle from entorhinal cortex), which reciprocally connect the hippocampal formation with a number of subcortical areas such as septal nuclei and mammillary bodies (figure 8).

Figure 8. Fimbria/fornix bundles

The axons of pyrmidal neurons in the hippocampus proper and subiculum gather at the ventral surface of the hippocampus proper as the alveus. Fibers in the alveus further converge to form the fimbria, a flattened ribbon of white matter attached to hippocampus proper. Finally the fibers in the fimbria form the fornix which detach from the hippocampus and arch ventrally.
Approximately 75% of fibers in the fornix terminate in the mamillary body, anterior nuleus of the thalamus and the midbrain regmentum; 25% of fibers in the fornix terminate in spetal nuclei, medial frontal cortex, anterior hypothalamus and ventral striatum.

Ninety percent of the cholinergic innervation of the hippocampus arises from the MSvDB. It is worthy to note that although the fimibria/fornix bundles are a major pathway for cholinergic innervation to the hippocampus, the fibers from MSvDB only account for a small part of the total number of fibers composing the fimibria/fornix bundles.

4. Mnemonic studies of cholinergic lesion induced by 192IgG-saporin

4.1 Central cholinergic system and memory/cognition

A substantial body of evidence suggests that the central cholinergic system plays an important role in memory and cognitive function (for review: 41, 43, 44). Based on the distribution of cholinergic neurons and their projections (figure 6), the central cholinergic system can be divided into two sub-systems: the basal forebrain cholinergic system consisting of basal forebrain nuclei and projections, and a brainstem pontine cholinergic system consisting of pedunculopontine tegmental (PPT) nuclei and lateraldorsal pontine tegmental (LDT) nuclei and their projections. The basal forebrain cholinergic system provides primary cholinergic innervations to neocortical, juxtallocortical (cingulated cortex) and allocortical sites (hippocampus, basolateral amygdala, and olfactory bulb). The basal forebrain cholinergic neurons play an important role in learning and memory,
whereas the brainstem pontine cholinergic system provides the main cholinergic innervations to the thalamus and other diencephalic structures. The pontine system regulates arousal and behavioral activation. Anatomic details of the basal forebrain cholinergic system are described above.

Interest in the function of the central cholinergic system, especially the basal forebrain cholinergic system greatly increased with demonstrations that depletion of cholinergic neurons of the basal forebrain and reduced cholinergic markers in the cerebral cortex and hippocampus were found in patients who died with Alzheimer’s disease (AD). With additional literature that described impairments in learning and memory following treatment with anticholinergic drugs, the cholinergic hypothesis/theory of memory dysfunction was proposed to explain the dementia associated with AD (67). Even though the cholinergic hypothesis/theory has been challenged in the pathogenesis of AD (45), it is well established that dysfunction of the cholinergic system contributes to the severity of cognitive and behavioral deficits, especially in the functional areas of memory and attention (46).

4.2 Animal model of selective cholinergic deficit induced by 192IgG-saporin

The immunotoxin, 192IgG-saporin (SAP), is a chemical conjugate of a mouse monoclonal antibody to the rat low-affinity nerve growth factor receptor (p75NGFR) and a ribosome-inactivating protein, saporin. The cell-surface antigen p75NGFR is primarily expressed in cholinergic neurons of basal forebrain and Purkinje neurons of the cerebellum. Therefore, direct infusion of SAP into basal forebrain nuclei produces a
selective lesion of cholinergic neurons, while sparing adjacent non-cholinergic neurons, thereby providing researchers with a powerful tool that is more selective than chemical, surgical or electrolytic lesion (48). Intracerebroventricular injection of SAP produces almost complete elimination of p²⁵NGFR-positive cells in rat brain, including cholinergic neurons of the basal forebrain and cerebellar Purkinje cells, but not cholinergic neurons of the brainstem (41). Microinjection of SAP into different areas of the basal forebrain dramatically reduces ChAT activity in either the neocortex or hippocampus. For example, microinjection of SAP into the MS/vDB produced a nearly 90% reduction in ChAT activity in the hippocampal formation (2) while microinjection of SAP into the NBM produced a 90% reduction in ChAT activity in forebrain (49). Permanent and selective removal of basal forebrain cholinergic neurons by SAP can provide an important animal model for understanding the role of forebrain and septal-hippocampal cholinergic function in behavior (48).

4.3 Assessments of memory impairment in rats with SAP induced selective septo-hippocampal cholinergic lesion

Selective cholinergic lesion of the medial septum (MS) is of interest to memory researchers because the MS provides the main cholinergic enervation of the hippocampus. Therefore, it was expected that a SAP model of selective loss of cholinergic neurons would be useful for studying memory impairments associated with AD (2). However, behavioral data from experiments that utilized SAP lesion of the basal forebrain were inconsistent in various behavioral assays/tasks of learning and memory. As a result, the function of MS cholinergic neurons in learning and memory was brought
into question (1). First, inconsistent or even contradictory results were found in a number of spatial memory tasks including the radial arm maze, T-maze and Morris water maze. A number of studies that used the radial arm maze (50, 51), T-maze (52; 53) and plus maze (54) reported that cholinergic lesion of the MS with SAP impaired spatial memory in rats. However, other studies that utilized the Morris water maze (55, 56; 57) or the radial arm water maze (58), reported that similar lesions spared spatial memory. Second, in contrast to the conflicting data produced with site-specific cholinergic lesions in the basal forebrain, severe impairment in learning and memory were consistently found following i.c.v. injection of SAP (51, 59, 60, 61). These impairments, though, may have been the result of the effects of SAP on motor performance or other variables rather than memory (2). As an example, cerebellar perkinje cells, which play an important role in motor coordination and balance, can be damaged through i.c.v. administration of SAP. Also, impairment could be the result of damage to cholinergic neurons outside of medial septum such as the lateral and ventral septal group (hDB,SI) projecting to the parahippocampal neurons of the entorhinal cortex and the nucleus basalis group (BAS/NBM) that project widely to the frontal, parietal, and temporal neocortices and amygdala. Therefore, as a whole, the data suggest that memory impairment induced by lesion of septal-hippocampal cholinergic neurons alone seems to be mild and task-selective compared to more extensive cholinergic lesions.

4.4 Potential reasons for inconsistent behavioral results in animal models of selective cholinergic lesions
Reasons for the inconsistent and/or contradictory results in response to cholinergic lesion could result from factors such as the size and degree of the lesion (62, 63, 69), the memory demands and/or complexity of specific protocols used in different laboratories (62, 64, 69), or the cognitive processes involved in specific memory tasks (65). For instance, even though the T-maze, radial arm maze and Morris water maze all test for spatial memory, the motivations and activities associated with each task are different. Motivations are commonly appetitive in the T- and radial arm mazes, but aversive in the Morris water maze. The former tasks are associated with reward and a lower stress level. In contrast, the Morris water maze is associated with higher stress levels and an aversive environment. Previous research in our laboratory demonstrated that the inconsistent results of studies in rats with septal-hippocampal cholinergic lesion may have been the result of different stress levels (66). The higher level of stress associated with the Morris water maze might make that test relatively insensitive to septal-hippocampal cholinergic deficits.

5. Tasks used to assess recognition memory

The most widely used tests of recognition memory are delayed matching/non-matching to sample tasks and novel object recognition tasks (22, 23). The delayed matching/non-matching to sample tasks requires that the subjects first be trained to learn the matching/non-matching rule with the aid of reward. In contrast, the novel object recognition task simply exploits an animal’s innate preference for novelty. The novel object recognition task is a simple test of recognition memory (the animal is not required to learn any rules to accomplish the task). It is, therefore, often referred to as a novel
object preference task (68) or spontaneous object recognition task (69). The advantages of the novel object recognition task include low stress level, no need for reinforcement training, low requirement for physical/motor activity, and intra-subject comparison available through repeated use of the same animals. Unlike many other animal behavioral tests, this task has the capacity to assess memory function with less potential confounds associated with various leaning processes.

5.1 Standard delayed matching/non-matching to sample paradigm

A sample is presented to the test animal and then, after a delay that ranges from a few seconds to several minutes, the sample object is presented again together with a novel object. A choice of the novel object is rewarded in the non-matching task; a choice of the sample object is rewarded in matching task. A Y-maze or T-maze is usually utilized in such tasks.

5.2 The standard novel object recognition paradigm

In the novel object recognition paradigm, two identical sample objects are presented and then, after a delay that ranges from a few seconds to several hours, the original sample object is presented again together with a novel object. Normally humans and other animals preferentially look at or explore the novel object, indicating that they recognize the familiar object.

In the above paradigms of recognition memory either working memory or reference memory for the object can be assessed depending upon the specific procedures. In fact,
apart from object recognition memory, paradigm variants of object recognition memory have been developed to assess spatial recognition memory or contextual recognition memory; e.g. delayed matching/non-matching to position in the T maze (52), spontaneous alteration in the plus maze (54), and object recognition tested in different rooms (72). In the present study, a modified version of a standard object recognition paradigm was developed to assess both long-term object recognition memory and spatial recognition memory (reference memory).
C. Hypothesis and specific aims

Hypothesis

Lesion of cholinergic neurons of the medial septum (MS) impairs object and/or spatial recognition memory in a novel object recognition paradigm.

Specific aim 1

Develop an object recognition paradigm and determine whether the surgical procedures affect recognition memory in this paradigm.

Specific aim 2

Determine whether lesion of cholinergic neurons of the MS by 192IgG-SAP impair retrograde object recognition memory.

Specific aim 3

Determine whether lesion of cholinergic neurons of the MS by 192IgG-SAP impair anterograde object recognition memory and anterograde spatial recognition memory.
II. EXPERIMENTALS

A. Materials and Equipments

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

Office
- Mellon Hall of Science, Duquesne University: Room 419

2. Animals
Sprague-Dawley male rats
Hilltop Lab Animal Inc. Scottdale, PA

3. Chemicals and drugs

192 IgG Saporin (SAP)
Chemicon, Temecula, CA

Artificial cerebrospinal fluid (CSF)
CMA Microdialysis, N.Chelmsford, MA

\[^{3}\text{H}]\text{ acetyl-CoA}
Sigma Inc., St. Louis, MO

Acetonitrile
Sigma Inc., St. Louis, MO

Acetyl-CoA
Sigma Inc., St. Louis, MO

Choline chloride (C\(_{5}\)H\(_{14}\)ONCl)
Sigma Inc., St. Louis, MO

Disodium phosphate (Na\(_{2}\)HPO\(_{4}\))
Sigma Inc., St. Louis, MO

EcoLume™ Liquid scintillation fluid
MP Biomedicals, Scottdale, OH

Econofluor scintillation cocktail
PerkinElmer Life And Analytical Sciences, Inc., IL
EDTA (Ethylene diamine Tetraacetic acid)  
Fisher Chemicals  
Halothane, USP  
Abbott Laboratories, North Chicago, IL

Ibuprofen Sodium  
Sigma Chemical Co., St Louis, MO

Pentobarbital Sodium  
Sigma Chemical Co., St Louis, MO

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

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

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

Sodium tetraphenylborone  
TCI America, Portland, OR

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

4. Materials

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

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

Disposable Scintillation Vials  
Fisher Scientific, Pittsburgh, PA

MLA Precision pipette tips (10, 20-250 and 20-1000µl)  
Fisher Scientific Pittsburgh, PA

Monosof Black Nylon Suture, 4-0, 3/8: 19mm  
Tyco Health Care, Norwalk, CT

Precision glide® disposable syringes needles (21, 23 and 26 gauge)  
Becton Dickson and Company, Franklin Lakes, NJ
Pipetters, Gilson Pipetman (P0.5, P10, P20, P200, P1000 and P5000)
Fisher Scientific Pittsburgh, PA

5. Equipment

Apparatus for novel object recognition built by our lab and consisting of the following items (figure 9)

- An open-field arena (45 cm×60 cm×60 cm) constructed of gray polyvinylchloride (PVC) plastic.
- Two floor lamps
- Video Camera
- Holding frame
- Sets of objects: mugs, light bulbs, coffee cup, saline bottles, syringes

1209 Rackbeta liquid scintillation counter
LKB Wallac

Brinkmann Bottle top Dispenser
Brinkmann instruments, Inc.

Stereotaxic frame
Stoelting, Wood Dale, IL

VirSonic 475 ultrasonic tissue disruptor

6. Computer Software

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

Microsoft word & Excel 2003
Microsoft Corporation, Orem, UT
Figure 9. Apparatus for Novel Object Recognition Task

Figure 9A: Open-field arena consisting of the floor board and walls.

Figure 9B: Objects consisting of blue porcelain mugs, white light bulbs, plastic saline bottles and black coffee cups, all of which were reconstructed so that they could be fixed on the floor board of the open field arena.

Figure 9C: Front view of the apparatus consisting of open-field walls, lamps and video-camera-holding-frame

Figure 9D: Side view of the apparatus consisting of lamps and video-camera-holding-frame
B. Methodology and Procedures

1. Animal Condition

All procedures involving the use of animals were in accord with standards established by the Animal Welfare Act and approved by the Institutional Animal Care and Use Committee of Duquesne University. Experimentally naive male Sprague-Dawley (SD) rats weighing between 250 and 275 g at the beginning of the study were purchased from Hilltop Lab Animals (Scottdale, PA) and housed in a temperature and humidity controlled facility with a 12:12 H light/dark cycle with food and water available ad libitum. The animals were allowed a minimum of five days to acclimate to the housing conditions before any experiments were performed.

2. Animal Surgery

Male SD rats weighing approximately 300 grams were anesthetized with pentobarbital (50 mg/kg: i.p., of a 50 mg/ml stock solution), shaved and placed in a stereotaxic frame (Stolting, Wood Dale, IL). After disinfecting the surgical area with iodine and isopropanol, an incision was made exposing the dorsal aspect of the skull. A small hole was drilled through which a stainless steel cannula was placed into the medial septum using coordinates from Bregma: AP + 0.2mm, L 0.0, DV -5.4mm from dura mater. Either SAP, 1 ug/ul or aCSF (sham control) 1 μl was infused into the medial septum over 5 minutes at a rate of 0.2μl/min. Following infusion, the cannula was left in place for 5 minutes to allow diffusion of the solution into the tissue. After the withdrawal of the cannula, the incision was sutured and Ibuprofen (1 mg/100g, i.p., from a 10 mg/ml stock solution)
solution) was administered for post-surgical analgesia. Rats were allowed to recover for two weeks before post-surgery training and retention testing.

3. Novel object recognition task

3.1 Apparatus for novel object recognition task

The novelty-preference task was conducted in an open-field arena (45 cm X 60 cm X 60 cm) constructed of gray polyvinylchloride (PVC) plastic. Three identical floorboards were prepared, one of which was bare and used during the acclimation phase. The other two had two caps used for attaching objects (figure 10), to maintain the position of objects during testing and between trials. The floors were removable from the walls to facilitate cleaning between trials. Dim diffusive indirect illumination from two lamps provided lighting for the apparatus. A video camera was positioned over the arena and the behavior of the rats was videotaped for later analysis. Stimulus objects included light bulbs, mugs, coffee cups, and saline bottles that were made of glass, porcelain, and plastic with different shapes and textures that could be easily cleaned. The objects were modified to be fixed easily to the floor by screwing to the cap on the floorboard. Rats were not able move the objects or hide in or under them. Objects were cleaned by tap water between the trials.

3.2 Novel object recognition task

As described in the Introduction, the novel object recognition task takes advantage of the natural tendency of rats to give more attention to a novel object than a familiar one. The test is selective for recognition memory and often referred to as objection recognition “test” rather than “task” (71). The procedure for this test consists of three phases:
acclimation, familiarization and testing. During the acclimation phase no objects are present. The purpose of this phase is to let the animal become familiar with the environment of the open field, thereby decreasing the stress level. During the familiarization phase, two identical objects were presented and the animal was allowed to freely explore the objects over a five-minute period. During the retention testing phase a familiar object and a novel object, or a familiar object in a novel location, were presented to the animal and the animal was allowed to freely explore the objects (novel and familiar / familiar and novel location) within a set time (three minutes). Animals were usually capable of perceiving the difference between novel and familiar objects and placement of the objects in familiar or novel locations. Rats naturally spend more time exploring a novel object or a familiar object in a novel location. The parameter measured during the test is the exploration time that the animal spent on each object. An exploration ratio was calculated for the time spent on the novel object or location divided by the total time spent exploring both objects.

\[
\text{The exploration ratio} = \frac{\text{Time (novel)}}{\text{Time (novel + familiar)}}
\]

The criteria to consider the animal to be engaged in object exploration included:

1. The distance between the object and animal within 4 cm
2. Exploring actions such as staring, touching, or sniffing the object
3. A total time spent exploring both objects greater than 10 sec in a 3-minute session.

Sitting on the object or touching the object without looking at it was not considered engagement. If the total time spent exploring both objects less than 10 sec, it may
indicate that the animal has some problems in either locomotive activity or attention, which will ruin the effectiveness of memory test.

The criterion to consider the animal to have recognition for old objects/location was an exploration ratio equal to or greater than 0.6, which is consistent with the mean exploration ratios for normal or control rats reported in most literature (68).

Figure 10. The Floors of the Open Field

Figure 10A: The caps of saline bottles were glued to the floor board; the mouth part of saline bottles were cut to build the base of the objects

Figure 10B: The sets of objects that were attached to the base.

Figure 10C: The floor board of the open field with the two caps in fixed position

Figure 10D: The floor board of the open field with two objects in fixed position
### 3.3 Timeline for behavioral experimental procedures

Three types of recognition memory were investigated: retrograde object recognition memory, anterograde object recognition memory, and anterograde spatial recognition memory. The timeline for the specific experiments are displayed in figure 11. This timeline was mainly based on the availability of the surgical techniques in the lab, general animal care and the characteristics of the clinic amnesia.

![Timeline for behavioral experimental procedures](image)

Figure 11. Timeline for behavioral experimental procedures

### 3.4 Procedures for retrograde recognition memory (figure 12A)

**Pre-surgery training**
- **Acclimation**: the rats were habituated to the arena by being allowed to explore the field for one 10-min session over two consecutive days without objects.
- **Familiarization**: the rats received one sample-exposure session per day for five consecutive days. During these sessions the rats were placed into the arena with two copies of the sample object and left to explore for 5 minutes.

**Post-surgery testing**

- **Re-acclimation**: after a 15 day delay, the rats were re-habituated to the arena and explored the field for one 5 min session without objects.

- **Retention testing** for object recognition memory: one day later after re-acclimation the rats were returned to the arena that contained the familiar object and a novel object for 3 minutes.

**3.5 Post-surgery procedures for anterograde recognition memory (figure 12B)**

- **Acclimation**: Rats were habituated to the arena by being allowed to explore the field for one 10-min session over four consecutive days without objects.*

- **Familiarization**: Rats received one sample-exposure session per day for two consecutive days. During these sessions the rats were placed into the arena with two copies of the sample object and left to explore for 5 minutes.

- **Retention testing for object recognition memory**: After a one-day delay, rats were returned to the arena which contained a copy of the familiar object and a novel object for 3 minutes.

- **Retention testing for spatial recognition memory**: One day after object memory retention testing, rats were returned to the arena for 3 minutes. The arena now contained two copies of the familiar object; one at the previous location, the other at a novel location.

*For the rats with pre-surgery training experience a one-day 10-min session is enough. For the rats without pre-surgery training experience a 10-min session per day over four consecutive days is needed.*
Diagram protocol for testing retrograde object memory

**Acclimation**
10 min/trial × 2 days

**Familiarization**
5 min/trial × 5 days
2 days later

**Surgery**
14 days later

**Retention testing for R. object memory**
3 min/trial

Diagram protocol for testing anterograde memory

**Acclimation**
10 min/trial × 4 day
(*10 min/trial × 1 day)

**Familiarization**
5 min/trial × 2 day
1 day later

**Retention testing for A. objects memory**
3 min/trial
1 day later

**Retention testing for A. Spatial memory**
3 min/trial

Figure 12. Procedures for recognition memory testing
Figure 12A: Procedures for retrograde object recognition memory
Figure 12B: Procedures for anterograde object and spatial recognition memory
A, B, X, and Y represent different sample objects: A, B, X and Y represent novel objects. In order to decrease bias introduced by objects/locations, half of the rats in each group were exposed to A/X as a sample object; the other half B/Y as a sample object. A similar rule was used for locations. * For the rats with pre-surgery training experience, one day 10-min session was satisfactory.
4. Animal behavioral studies

The study was divided into three parts shown in the below, totally sixty animals were used.


**Part 2**: The effect of SAP lesion on retrograde object recognition memory.

**Part 3**: The effects of SAP lesion on anterograde object recognition memory and anterograde spatial recognition memory.

5. ChAT (Choline Acetyltransferase) Assay

To confirm the SAP lesion of the medial septum and thus cholinergic deficit of the hippocampus, a ChAT assay was performed. After all the behavioral tests animals were processed for the quantification of brain ChAT activity within weeks. Animals were anesthetized with 5% halothane in oxygen and decapitated. Then brains were removed from the skull, and tissues from the frontal cortex (FC), medial septum (MS) and hippocampus (H), were dissected and frozen at -80°C. A radiochemical assay to quantify ChAT activity was performed (52, 53). In brief, on the day of the assay, frozen tissues were thawed at 4°C and dissociated by sonication in a medium containing EDTA (10mM) and Triton X-100 (0.5%) and diluted to 10 mg tissue/ml. An aliquot from each sample was used for the determination of total protein. Three 5μl aliquots were incubated for 30 min in a medium containing 0.25 mM [H³]-Acetyl-CoA, 50 mM sodium phosphate buffer (pH 7.4), 300 mM sodium chloride, 10 mM choline chloride, 10 mM EDTA and 0.2 mM physostigmine sulfate. The reaction was stopped by adding 4 ml of
10 mM sodium phosphate buffer (pH 7.4) followed by the addition of 1.6ml of acetonitrile containing 5mg/ml tetraphenylboron in scintillation vials. The amount of $[^3]$H–acetylcholine produced was determined by adding 8 ml of EconoFluor scintillation cocktail and counting total cpm in the organic phase using an LKB beta-counter. Background was determined using identical tubes to which no sample was added. For each sample, three reaction tubes containing sample were averaged and the difference between total cpm and background cpm was used to estimate the total amount of ACh produced per sample. ChAT enzyme activity was expressed as ACh produced/h/µg protein.

6. Statistical Analysis

All analyses were performed using GraphPad Prism 3.02. Differences in ChAT activity in the hippocampus and frontal cortex of SAP treated and control animals were compared using a two-way ANOVA with a Bonferroni post-hoc test. Differences in the mean exploration ratio between groups were compared with either Student t-test or one-way ANOVA depending on the number of groups. Significant differences between groups were interpreted as $p < 0.05$
III. Results

ChAT Assay

Effects of SAP infusion in the MS on ChAT Activity in frontal cortex and hippocampal formation

In order to verify that the surgical infusion of SAP into the MS actually resulted in lesion of the septal-hippocampal tract, a ChAT assay was performed for fifty-two* animals after the completion of the behavioral studies. The ChAT activity in the hippocampus for nine rats in the SAP group were found to be normal (the criteria for considering the SAP rats with the normal hippocampal ChAT activity was the range of ChAT activity of the control group and statistical outliers). Cholinergic neurons in the MS for these nine rats were not lesioned at all or only partially lesioned. In view of the purpose of this study (the role of septal-hippocampal cholinergic deficit), all the data for these rats, therefore, were excluded from later data analyses. Two-way ANOVA with a Bonferroni posthoc test for individual comparisons between groups was performed for the ChAT activity of the rest animals with more complete lesion. The effects of SAP lesion on levels of ChAT activity in the hippocampus and frontal cortex tissue are summarized in table 1 and shown in figure 14. Two-way ANOVA revealed a significant effect of “treatment” \[ F(1,82) = 332.8, p < 0.0001 \], a significant effect of “brain area”\[ F(1,82) = 25.02, p < 0.0001 \] and a significant interaction “between treatment and brain area” \[ F(1,82) = 86.52, p < 0.0001 \]. The treatment effect indicated that the SAP infusion in the MS caused a significant decrease in ChAT activity in the hippocampus and frontal cortex relative to control animals. The significant effect of “brain area” indicates that ChAT activity in the hippocampal formation is higher than that in frontal cortex. The significant
interaction between treatment and brain area indicates that the effects of SAP treatment on ChAT activity in the hippocampal formation and the frontal cortex are different, in other words, the decrease of the ChAT activity in the hippocampal formation is severer than that in the frontal cortex. Bonferroni *post hoc* tests revealed that SAP treatment caused a significant decrease in ChAT activity in both the hippocampal formation and the frontal cortex (*p* < 0.0001), but the decrease of ChAT activity in the hippocampus was 85.8%, compared to 30.8% in the frontal cortex.

**Figure 13.** The effect of SAP infusion in the MS on ChAT activity in the hippocampus and frontal cortex for the lesioned rats. The bars represent the mean value of ChAT activity and the standard error of mean for groups. The animal numbers for each group are shown in table 1.
Table 1. The effect of SAP infusion in the MS on the ChAT activity of hippocampus and the frontal cortex for the lesioned rats

<table>
<thead>
<tr>
<th>pmol Ach produced hr/ug protein</th>
<th>Control (n=26)</th>
<th>SAP (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Means ± s.e.m.</td>
<td>Range</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>38.93 ± 1.21</td>
<td>29.61-55.42</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>33.76 ± 0.96</td>
<td>24.81-41.26</td>
</tr>
</tbody>
</table>
B. Animal Behavioral Studies

Part I: The effect of surgery procedures on recognition memory

To determine whether anesthesia and surgery could affect memory function, rats without surgery (n=10) and surgical rats infused with artificial cerebrospinal fluid (aCSF; n = 9) were tested first. Figure 15 shows the mean exploration ratios of both groups for the three types of recognition memory. Bars represent the mean ± SEM. The data were collected from two independent experiments for anterograde recognition memory, but only one for retrograde recognition memory because the data from the first experiment for retrograde recognition memory was invalid due to the imperfect protocol. Therefore, only the data from the second experiment with improved protocol was used for retrograde object recognition memory. Student’s T-test was used to compare the two groups in retrograde object recognition memory (p = 0.914); one-way ANOVA was used to compare the four groups in anterograde object and spatial recognition memory (p = 0.879). There was no significant difference between the mean exploration ratios for the non-surgery group and the CSF group. The mean exploration ratios were between 0.6 and 0.7, which is consistent with data for normal animals reported in the literature (68, 71, 72 and 74). This result indicates that the surgery had no effect on memory function and that the methodology for object and location recognition was satisfactory.

Part II: The effect of SAP treatment on retrograde object recognition memory

In order to measure retrograde recognition memory, surgery was performed two days after training. All rats were trained in the object recognition task and then randomly divided into two groups: surgery with SAP infusion and surgery with aCSF infusion.
Fourteen days following surgery a re-acclimation session was administered and the following day retention for object recognition memory was tested. Effects of SAP treatment on the mean exploration ratio (MER) and mean total exploration time (MTET) for retrograde object recognition memory are shown in figure 16. Bars represent the mean ± SEM. Data were collected from three independent experiments. The MER for the SAP group was similar to that of the control group; (0.68±0.04 vs 0.67±0.03; \(p=0.888\)). There was no significant difference in the MTET for the SAP group and the control group (46.4±2.8 seconds vs 52.1±3.2 seconds, \(p=0.275\)) by Student T-test. These results indicate that SAP infusion in the MSvDB did not impair retrograde object recognition memory.

**Part III: The effect of SAP treatment on anterograde recognition memory**

Three days after post-surgery retention testing for retrograde object recognition memory, object recognition testing for anterograde object and spatial recognition were performed. The results of SAP treatment on anterograde object recognition memory and anterograde spatial recognition memory are shown in figures 17 and figure 18, respectively. Bars represent the mean ± SEM. The data were collected from six independent experiments.

1. **Effects of SAP treatment on anterograde object recognition memory**

Nine animals in the SAP group were excluded from data analysis due to the failure of SAP to produce a cholinergic lesion, verified by ChAT assay of the hippocampus in those animals. As shown in figure 17, the MER of the SAP group (n=17, 0.67±0.04) was similar to that of the control group (n=29, 0.66±0.02). The MTET for both groups was
also similar (55.2±4.7 vs 52.7±2.3 sec). There was no significant difference between the SAP treated and the control group in MER ($p = 0.866$) or MTET ($p = 0.604$) by Student T-test. These results indicate that SAP lesion of the MS spared anterograde object recognition memory.

2. **Effects of SAP treatment on anterograde spatial recognition memory**

Two additional animals in the SAP group were excluded from data analysis because the total exploration time was less than 10 sec, which indicated that the animal might have defect in attention or locomotive activity. As shown in the figure 18, the MER of the SAP group was significantly decreased compared to the control group (0.51 ± 0.04 vs 0.62±0.02; $p = 0.0081$). But the MTET for both groups was similar (45.1±4.8 seconds vs 39.7±2.5 sec; $p = 0.279$). These results indicate that SAP lesion of the MS impaired spatial recognition memory and this impairment was not due to an attention deficit.
Figure 14: The effect of surgery procedures on recognition memory

Figure 14A: The effect of surgery procedures on retrograde object recognition memory.

Figure 14B: The effect of surgery procedures on retrograde object recognition memory.

Vertical axis represents the mean exploration ratio. Bars represent mean ± SEM. Normal or NOR stands for the normal control group. CSF stands for the artificial cerebrospinal fluid surgical infusion group. Object represents object recognition memory; Location represents spatial recognition memory. The number in parentheses represents the number of rats in each group. The data in figure 15A was analyzed with Student T-test. The data in figure 15B was analyzed with one-way ANOVA. No significant differences were detected between the groups in either figure. The mean exploration ratios for all the groups were above 0.6. That indicates that the surgery for the septal infusion had no effect on memory function and the paradigm for object and spatial recognition worked well.
Figure 15. Effects of SAP treatment on retrograde object recognition memory.

A: Effects of SAP treatment on the mean exploration ratios (MERs).

B: Effects of SAP treatment on the mean total exploration time (MTET).

In the figure 16A, the vertical axis represents the mean exploration ratio. In figure 16B the vertical axis represents the mean total exploration time (seconds) for novel and familiar objects. The number in parentheses represents the number of rats in each group. Bars represent mean ± SEM. There was no significant difference between the SAP treated and the control group in MER ($p=0.888$) and the MTET ($p=0.275$). The mean exploration ratios for both groups were above 0.6 which indicates that the SAP treatment had no effect on retrograde object recognition memory.

Figure 16. Effects of SAP treatment on anterograde object recognition memory.

A: Effects of SAP treatment on the mean exploration ratios (MERs).

B: Effects of SAP treatment on the mean total exploration time (MTET).

In figure 16A, the vertical axis represents the mean exploration ratio. In figure 16B the vertical axis represents the mean total exploration time (seconds) for novel and familiar objects. The number in parentheses represents the number of rats in each group. Bars represent mean ± SEM. There was no significant difference between the SAP treated and the control group in both the MERs ($p=0.866$) and the MTETs ($p = 0.604$). The mean exploration ratios for both groups were above 0.6, that indicated that the SAP treatment had no effect on anterograde object recognition memory.
Figure 17. Effects of SAP treatment on anterograde object recognition memory.
A: Effects of SAP treatment on the mean exploration ratios (MERs).
B: Effects of SAP treatment on the mean total exploration time (MTET).
In figure 17A, the vertical axis represents the mean exploration ratio. In figure 17B the vertical axis represents the mean total exploration time for both novel and familiar objects. The number in parentheses represents the number of rats in each group. Bars represent mean ± SEM. There was a significant decrease in SAP treated compared to the control group in both the MER ($p = 0.0081$) but not MTET ($p=0.279$). These results indicate that SAP treatment impaired anterograde spatial recognition memory.
IV. DISCUSSION

The SAP lesion—the results of ChAT assay

The selective cholinergic neurotoxin 192IgG-saporin (SAP) was utilized to produce a cholinergic deficit in the hippocampal formation via microinjection of SAP into the MS. The dose and the coordinates for the MS were based on the previous study in our lab, which had been shown to be effective and appropriate (52). However, the stereotaxic surgery for infusion of SAP is highly challengeable and individual-dependent; therefore, the confirmation of the SAP infusion into the appropriate position is always necessary. The results of ChAT assay indicated that the hit rate of SAP lesion was around 70% (17/26); in other words, nine animals (30%) were not lesioned well. In order to fulfill the goal of this study, the data aroused from these rats have to be excluded. After exclusion of these non-lesioned or partially-lesioned rats, the ChAT activity in the hippocampus for lesioned rats showed a normal distribution and the mean value of ChAT activity in the hippocampus for lesioned rats was 85.8% decrease compared with that for non-lesioned control rats. The mean value of ChAT activity in the frontal cortex for lesioned rats was also significant decreased though it was only 30.8%. In view of the fact that the MS and vDB as a functional unit provides a small portion of cholinergic enervation to the frontal cortex in addition to the hippocampal formation, the above results indicates that the size of SAP lesion might be deeper and broader than we planned, but still appropriate. Since the decrease of ChAT activity in frontal cortex may have an effect on attention, which could confound with memory function, the total exploration time on both objects were assessed to detect attention deficit.
Memory impairment—the results of animal behavioral studies

An object exploration paradigm was used to address the role of the septal-hippocampal cholinergic tract in three types of recognition memory: retrograde object recognition, anterograde object recognition, and anterograde spatial recognition. In retrograde recognition memory only object recognition memory was tested, because impairment in object recognition memory was expected in the SAP lesion group according to several studies (74, 75). However, in the present study, contrary to our expectations, SAP treatment did not result in impairment of retrograde object recognition memory. Several possibilities could account for this result including the drawbacks in the methodology; (e.g. the two-day time delay between training and surgery is actually not perfect time to affect the process of memory consolidation), or the septal-hippocampal cholinergic tract might not be necessary for retrograde object recognition memory. Considering the limitations from the stereotaxic surgery, the results from early phase of this study and the purpose of this study, we decided to stop the investigation of retrograde object recognition memory in later phase of study.

Anterograde object and spatial recognition memory were the focus of this study. Both control and SAP lesion groups preferentially explored the novel object introduced into a familiar location, indicating recognition of the object as novel. However, when a familiar object was moved to a novel location, control rats indicated recognition of the change in location by increasing the exploration time of the displaced object, but the SAP lesion group did not. This result suggests that the septal-hippocampal cholinergic tract may not
be necessary for anterograde object recognition memory, but does function in anterograde spatial recognition memory.

Our findings are consistent with a number of reports that the hippocampus is a critical structure for spatial memory, but not object recognition memory (72, 76-83). In addition, our findings are consistent with studies of the effect of selective cholinergic lesion of the medial septum that result in deficits in spatial or spatial recognition memory (50-52, 54, 55, 57, 60, 84-86). Most interestingly, these finding are consistent with a clinical phenomena shown in the development of AD, which is that the spatial memory (including object location and navigation) is usually more vulnerable than other types of memory (e.g. face recognition) in the early phase of AD.

Our study indicates that the degeneration of the septo-hippocampal cholinergic neurons may be responsible for the early symptoms in AD. It is worthy to note that the dissociation observed between the effects of septal cholinergic lesion on the two forms of anterograde recognition memory is not due to cholinergic function per se but the different role of hippocampus in object and spatial recognition memory.

**Hippocampus and recognition memory**

The hippocampus has long been known to be critical in memory and learning, e.g. memory consolidation (87, 88), descriptive memory (89), and especially in spatial memory (90). However, the role of the hippocampus in recognition memory has been controversial. On the one hand, hippocampal lesions have been reported to impair recognition memory (21, 91); on the other hand, it has been reported that recognition memory is spared with hippocampal lesion (92, [for review see 73]). Hippocampal lesion size, representational demands of the task and the specific protocols of testing were
suggested to play a role in the contradictory results (77, 91, 93). Interestingly, a number of studies report that hippocampal lesions produce deficits in spatial but not object recognition memory (72, 76-79, 80-83). Recent reviews (18, 22) document accumulating evidence that supports a two-component theory of recognition memory: 1) a sense of familiarity and 2) recollection of the stimulus in the context of other information associated with the previous experience. The hippocampus is critical for recollection but not familiarity, whereas the perirhinal cortex is involved in the process of familiarity. This view was supported by the differential effect of selective perirhinal cortex lesion on spatial and object recognition memory reported by Barker et al. (94). Excitotoxic lesion of the perirhinal cortex of rats by NMDA caused impairment of object recognition memory but spared spatial recognition memory. Therefore, one explanation for the variable results of different studies of the effects of hippocampal damage on recognition memory may be that the demands for the two processes involved in recognition memory differ across paradigms with the hippocampus supporting only one of the two processes, recollection (95). For the object recognition task in the current study, familiarity alone was sufficient to detect object novelty since the familiar object was visible in the open field but recollection in addition to familiarity may be required to detect spatial novelty because the animal has to recollect the context of where the object was originally located. When the animal failed to recollect the contextual information about previous location, this would manifest itself as a failure to increase exploration time of the object in the novel location.
Recognition memory and spatial memory are often considered to be two distinct memory functions (91), but actually, they involve overlapping processes classified by different perspectives. Recognition is based on recall. Spatial memory is based on the context of the memory. Therefore, in terms of function, recognition memory and spatial memory are not identical but interconnected. Most learning and memory tasks tested in animals are based on recognition memory. The performance of an animal is dependent on the ability to recognize a previously experience such as object, odor, foot shock, sound, location or environment. Spatial memory is related to object location and/or navigation. Spatial memory can be spatial recognition memory (e.g. detecting the changes of the position of the furniture in an office), cued-recalled spatial memory (e.g. navigation in an unfamiliar environment with some known landmarks) or free-recalled spatial memory (e.g. making a map of campus) depending on the particular testing methodology. Most spatial memory functions tested in animals are a type of spatial recognition memory or are based on spatial recognition in which recollection is involved. This may be the reason that the hippocampus always appears to be critical for spatial memory.

Data from functional magnetic resonance imaging (FMRI) in humans (95) indicate that the hippocampus selectively supports recollection memory—the retrieval of the contextual details of episodic memories. In that study, subjects performed a “remember-know” task. During scanning the subjects were required to classify their memory as either episodic (remember) or based on familiarity (know). Activity in the hippocampus increased only when retrieval was accompanied by a consciousness of the learning episode. Hippocampal activity did not increase for items recognized based on familiarity.
Further, a clinical report by Bowles et al. (98) provides additional support for this theory. A patient with anterior temporal-lobe resection that destroyed the perirhinal cortex but spared the hippocampus exhibited impaired familiarity but preserved recollection.

Even though episodic memory cannot be directly assessed in animals, one important feature of episodic memory is the retrieval of spatial and/or temporal context that can be assessed in animals using contextual learning protocols (99). A study reported by Eacott & Norman (100) revealed a dissociative effect between object novelty and spatial novelty in animals with a hippocampal lesion in a variant of a spontaneous novelty exploration task where the initially presented object was moved to a new location or to a new environment during the test phase. A functional double dissociation between the effects of peri-postrhinal cortex and hippocampal lesion reported by Winter et al. (83) revealed that rats with hippocampal lesions were impaired in a spatial memory task relative to the control rats and the rats with peri-postrhinal cortex lesion, whereas rats with peri-postrhinal lesions were impaired in an object recognition test relative to the control and the hippocampal lesion group. These findings provide strong support for the results of the present study: SAP lesion targeting the cholinergic innervations of the hippocampal formation impaired spatial recognition memory but spared object recognition memory. These results indicate that cholinergic neurons of the MS play an important role in hippocampus-related memory function.

The medial temporal lobe and the detection of the novelty in recognition memory
According to recent reviews by Kumaran and Maguire (96), there are three categories of novelty: stimulus novelty (e.g. novel object), associative novelty, (items that are familiar but appear in a novel configuration, e.g. a familiar item in a new location), and contextual novelty (e.g. familiar items in a new environment.) Different categories of novelty utilize different structures of the medial temporal lobe. Stimulus novelty is processed by the perirhinal cortex, while associate novelty is mainly processed by the hippocampus (96, 97). According to this view, the anterograde object recognition and anterograde spatial recognition tested in the present study are categorized as stimulus novelty and associative novelty, respectively. The results of our study are consistent with these previous results.

The present study demonstrated that hippocampal-dependent spatial recognition memory was sensitive to the loss of cholinergic neurons projecting from the MS/vDB. The findings of this study are consistent with other studies with nonselective lesions of either the MS (80) or hippocampus (72-83). Further, these results are consistent with recognition memory processes involving different regions of the medial temporal lobe: hippocampal and perirhinal cortex. A study by Winters and Bussey (101) reported that selective removal of cholinergic input to perirhinal cortex disrupts object recognition but not spatial memory in rats (101). This study provides complementary support to our study that cholinergic neurons are important in memory and learning and the view that the effect of cholinergic lesion depends on the particular brain structure and specific behavioral task.

**Selective cholinergic lesion of the MS/vDB and spatial recognition memory**
Our findings are also consistent with a number of reports that selective cholinergic lesion by SAP in the MS/vDB impaired spatial memory in the radial arm maze (50, 51; 57, 63, 86), T-maze (52, 53), plus-maze (54; 55), and the cone-field task (102). It is worthy of note that most studies that show impaired memory following cholinergic lesion of the basal forebrain tested anterograde memory. The reason we emphasize this point is because a number of studies in which SAP lesion of the MS did not impair performance in the radial maze (103), T-maze (104), or other spatial task (105), were tests of retrograde memory. However, one important factor associated with retrograde memory is that the time between task training and creation of the lesion was not addressed clearly in these papers.

Another aspect of the literature that should be noted is that a number of studies investigated the role of cholinergic neurons of the septal-hippocampal tract and reported negative results in the Morris water maze (55, 56, 57) or some other task related to swimming (58). It is possible that a swim task may not be a sensitive test for memory deficits related to lesion of septal-hippocampal cholinergic neurons. As an example, one report found that in the same set of rats cholinergic lesion in the MS caused impaired performance in the radial arm maze but not the water maze (57). Also, in a water version of the radial arm maze selective cholinergic lesion did not impair memory performance (58, 106). It is possible that the difference in sensitivity between a water maze and a land-based maze (e.g. radial maze, T maze etc.) is the level of stress and the amount of physical effort involved in the task. First, the greater level of stress associated with a swimming task may lead to the use of additional neuronal tracts to solve the problem.
Fitz et al. (66) demonstrated that the introduction of an aversive stimulus could attenuate the impairment of acquisition in a delayed match to position T-maze task following selective cholinergic lesion of the septal-hippocampal pathway. Second, depletion of ACh in the septal-hippocampal tract may impair spatial memory performance by reducing dentate gyrus (DG) neurogenesis (107). It is well known that exercise can facilitate brain function by increasing DG neurogenesis (108). Thus, physical activity associated with the Morris water maze may mask the effect of cholinergic lesion of the MS.

**Advantages of the present study**

A wealth of evidence indicates that the effects of hippocampal lesions depend upon the complexity of the task (53, 62, 64). As the complexity of the task increases the number of confounders/variables increases, which then increases the functional demands on the hippocampus. The present study utilized a modified version of an object recognition task with a minimum number of variables and hence is a relatively pure test of recognition memory. The novel methodology of this study is simple by combining two tasks into one to detect the effects of cholinergic lesion of the MS on both object recognition and spatial recognition memory in the same set of rats. The potential biases associated with the intensity of light source, the objects and their locations *per se* were limited by taking those factors into consideration in the experimental design. The performance of the control group was consistent with other studies that used a novel object recognition test.
V. CONCLUSIONS

1. The septal-hippocampal cholinergic neurons are important for a hippocampal-dependent memory function: spatial recognition memory, but not the hippocampal-independent memory function: object recognition memory, which provided the additional evidence for a two-component theory of recognition memory.

2. The septal-hippocampal cholinergic neurons may be responsible for mild memory impairment shown in the early phase of AD: impaired spatial memory, but spared face recognition memory.

3. The pathologic model resulting from the septal-hippocampal cholinergic lesion with SAP is not adequate for severe memory impairment in late phase of AD but good for mild memory impairment in the early phase of AD; the modified object recognition paradigm developed in this study is a novel and effective behavioral assay to detect this mild memory impairment induced by this model.

4. Combined with the behavioral assay established in this study, the SAP lesion of the septal-hippocampal tract can be used as a simple and effective tool to screen the potential drug candidates for memory improvement.

5. This modified novel object recognition task can also be used to test the pathological model for memory impairments in AD induced by factors other than cholinergic neurons.
VI. References


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69


