The Development of an Object-Recognition Task for Rats and the Evaluation of the Internal Validity of the Novel-Object-Preference Test

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Abstract

The Development of an Object-Recognition Task for Rats and the Evaluation of the Internal Validity of the Novel-Object-Preference Test

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Object-recognition—the ability to discriminate the familiarity of previously presented stimuli—is assessed in laboratory rats using the delayed nonmatching-to-sample (DNMS) task and the novel-object-preference (NOP) test. The DNMS task provides a fairly precise measure of a rat's object-recognition abilities, however, it suffers from certain drawbacks. In particular, rats require extensive training and it cannot be used to assess memory for objects following periods lasting longer than several minutes. For these reasons, most researchers have abandoned it in favour of the NOP test. The NOP test is easy to use, as it relies on measuring a rat's natural tendency to spend more time investigating a novel object over a familiar one when both are presented in a familiar context. Some concerns have been raised, however, regarding the internal validity of the NOP test. Accordingly, the goal of the present thesis was to develop a new object-recognition task that addresses the known limitations of the existent tests. A secondary goal of the thesis was to evaluate rats' performance on the new task to that on the NOP test as a means to validate the latter. The first experiment describes rats' performance on the new task –the modified DNMS (mDNMS) task. Rats required significantly fewer trials to learn the nonmatching rule compared to conventional DNMS tasks, and their scores showed good test re-test reliability. The same rats' exhibited significant novelty-preference scores on the NOP test, however their scores showed poor test re-test reliability and were not significantly correlated with mDNMS scores. The latter finding suggests that the two tasks may not tax similar underlying cognitive processes. In the experiment presented in Chapter 3, memory for objects was assessed following delays lasting 72 hr, 3 weeks, and ~45 weeks on both the mDNMS task and NOP test. Rats successfully discriminated between novel and sample objects on the mDNMS task following all three delays, however, the same rats failed to exhibit significant novelty preferences following all three delays on the NOP test. These findings reveal that the mDNMS task can be used to assess long-term memory for objects, and that a failure to exhibit a novelty preference may not necessarily reflect

the status of object-recognition memory. Next, we assessed rats' performance on the mDNMS task and NOP test following surgical lesions made to either the hippocampus (HPC) or perirhinal cortex (PRh)—two brain areas implicated in object-recognition memory. Neither HPC nor PRh lesions failed to disrupt performance on the mDNMS task, but rats with PRh lesions failed to display a novelty preference on the NOP test. The discrepancy in the PRh rats' performance on both tasks further adds to concerns regarding the internal validity of the NOP test, such that a lack of novelty preference is not necessarily indicative of an object-recognition memory impairment. The final experiment focused on refining the mDNMS task to include an additional behavioural measure—*latency to make a choice*. We incorporated a Go/No-go procedure and found that latency to make a choice provided a more sensitive measure of object-recognition memory than choice-accuracy on the test. Collectively, these findings confirmed the utility of the mDNMS task as a means to gauge object-recognition memory in rats. The results also highlight the limitations of the NOP test, and raise concerns regarding the internal validity of it as a means to measure object-recognition abilities in rats.

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Contributions of Authors

Chapter 2

In collaboration with Dave Mumby, I developed the experimental design. I collected the behavioural data in collaboration with Amanda Simundic, Frank Mossa, Devon Merza, Lianne Trigiani, Joelle Ziadé, and Jonathan Cuthbert. I coded the behavioural data with assistance from Frankie Mossa and Amanda Simundic. I performed the statistical analyses, and wrote the manuscript with the assistance of Dave Mumby.

Chapter 3

In collaboration with Dave Mumby, I developed the experimental design. I collected the behavioural data with some assistance from Francis Carter and Julian Alvarez-Barkham. I coded the behavioural data and performed the statistical analyses. I wrote the manuscript with the assistance of Dave Mumby.

Chapter 4

In collaboration with Dave Mumby, I developed the experimental design. I collected the behavioural data in collaboration with Joelle Ziadé, Amanda Simundic, and Pawel Jastrzebski. Joelle Ziadé performed the majority of the surgeries under my supervision, and assisted me with the aftercare of the rats. I coded the behavioural data with assistance from Joelle Ziadé and Amanda Simundic. I conducted the histological analyses with assistance from Amanda Simundic. I performed the statistical analyses, and wrote the manuscript with the assistance of Dave Mumby.

Chapter 5

In collaboration with Dave Mumby, I developed the experimental design. I collected and coded the behavioural data in collaboration with Megan Chad and Vanessa Moman. I conducted the statistical analyses and wrote the manuscript with the assistance of Dave Mumby.

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List of Abbreviations

AMY	Amygdala
ANOVA	Analysis of variance
CA	Cornu Ammonis
CI	Confidence interval
cm	Centimeter
DNMS	Delayed nonmatching-to-sample
EC	Entorhinal cortex
HPC	Hippocampus
hr	Hour
ICC	Intraclass correlation
IP	Intraperitoneal injection
lx	Lux
mDNMS	modified delayed nonmatching-to-sample
MTL	Medial temporal lobe
NMDA	N-methyl-D-aspartic acid
NOP	Novel-object preference
NOR	Novel-object recognition
PH	Parahippocampal
POR	Postrhinal cortex
PRh	Perirhinal cortex
PVC	Polyvinyl chloride
s	Seconds
SC	Subcutaneous injection
SEM	Standard error of the mean
SOR	Spontaneous object recognition

Chapter 1: General Introduction

A central feature of psychology research involves using animals as model systems for studying general processes, such as learning and memory. A goal of this research is to uncover the underlying biological mechanisms of these processes and to ultimately solve specific problems (e.g., develop treatments for neurodegenerative diseases). When using animals to examine internal constructs such as memory, researchers must make inferences about these constructs based on the animal's behaviour. Accordingly, an essential feature of any research design is the inclusion of a suitable behavioural task—one that can accurately estimate the specific construct under investigation. Both the quality, and hence, validity of the research findings are dependent on this factor. The goal of this thesis is to develop and assess the utility of a new object-recognition task for rats.

Object-recognition memory is the ability to discriminate the familiarity of previously encountered objects. It is a fundamental memory ability that most people engage hundreds of times each day –often with little or no conscious awareness, but sometimes accompanied by explicit recollection of one or more specific episodes on which a previous encounter occurred (Aggleton & Brown, 1999).

Laboratory rodents also distinguish between objects they have previously encountered and ones they have not. The extent to which object-recognition memory involves similar cognitive processes in rodents and humans is not entirely clear. Still, numerous studies in rats and mice have examined how drugs, brain lesions, or other treatments affect performance on tests that are presumed to be effective for discriminating between animals with different object-recognition capabilities (Brown, Warburton, & Aggleton, 2010; Warburton & Brown, 2015; Winters, Saksida, & Bussey, 2008).

Two behavioral paradigms have been used to assess object recognition in rats: *delayed nonmatching-to-sample* (DNMS), using trial-unique[1] stimuli, and *novel-object preference* (NOP). On the DNMS task, a sample object is briefly presented and after a retention interval, the sample is presented again along with a novel object. The rat receives a reward if it selects the novel (nonmatching) object. Different sample and novel objects are used on each trial, so reliably

[1] "Trial-unique" indicates that stimulus items do not recur *within* a test session, however, they may recur throughout testing but over widely distributed points in time. The term is used to distinguish from earlier delayed-response tasks that had recurring stimuli within a session.

accurate performance requires that rats can recognize the sample objects. Memory demands are manipulated by varying the retention interval or the number of objects to remember on each trial.

Although the DNMS task provides a fairly precise estimate of a rat's object-recognition abilities, the existing DNMS procedures share some drawbacks in common. Specifically, they are difficult to employ and require extensive training of the rats. Consequently, most investigators who sought to study object-recognition in rodents abandoned the DNMS task in favor of the NOP test, mainly because the latter is relatively easy to employ. The NOP test takes advantage of a rat's natural tendency to investigate novel objects more than familiar ones when both are presented in a familiar environment (Berlyne, 1950; Besheer & Bevins, 2000; Ennaceur & Delacour, 1988). On conventional NOP tests, a rat is placed in an arena where it is allowed to investigate two identical objects for a few minutes. The rat is then removed for a retention delay, after which it is returned to the arena, where there are now two new objects –one is identical to the sample and the other is novel. On the test, if the rat spends more time investigating the novel object compared to the sample, it is inferred that the rat recognizes the sample object.

Despite the practical advantages of the NOP test however, some recent observations have raised concerns about the internal validity of it as a gauge for object-recognition abilities. Specifically, it is unclear to what extent the degree of novel-object preference reflects the persistence or accuracy of the memory for the sample object.

The primary goal of this thesis work was to develop a new object-recognition task, one that would address the known limitations of the extant tasks, and could not be subjected to the same criticisms of that of the NOP test. We decided to develop a modified DNMS (mDNMS) task—one that would incorporate the advantageous features of conventional DNMS tasks, yet would be easier for rats to acquire and less difficult for experimenters to use. The thesis experiments consisted of the following: 1) the development of the mDNMS task, 2) measuring performance on the mDNMS task following long retention intervals ranging from days to one year, 3) using the mDNMS task to examine the effects of discrete lesions made to brain areas hypothesized to be critical for object-recognition memory, and lastly 4) the refinement of the mDNMS task to further improve the procedure.

A secondary goal of the thesis was to evaluate the utility of the NOP test as a measure of object-recognition memory by validating it against the mDNMS task. Thus, within each experimental chapter, the rats were also tested on the NOP test and their scores were compared

to scores obtained on the mDNMS task.

Overall, the findings reveal that the mDNMS task is an effective tool for measuring both short and long-term object-recognition memory, and that rats can acquire the task much faster than conventional DNMS tasks. Moreover, the results raise concerns regarding the manner in which NOP data are interpreted, namely, that novelty-preference scores should not be uncritically assumed to reflect the status of object-recognition memory. These findings are important because they demonstrate the advantages of using an alternative approach to the existing object-recognition tests, while also revealing major problems concerning the internal validity of the NOP test as a means to gauge object-recognition abilities in rats.

The following sections describe the history and rationale for assessing object-recognition memory in rats, followed by a discussion on the shortcomings of the existing tasks. It begins with a description of brain-damaged-produced amnesia in human neuropsychological patients (Section 1.1). This is followed by a historical description of the progression from nonhuman primate models of human amnesia to more focused research on the biological basis of one facet of recognition memory—object-recognition memory (Section 1.2). Next, a historical account is provided on the development of object-recognition tasks for use with rats, followed by a description of the drawbacks of the extant tasks (Section 1.3). Lastly, Chapter 1 ends with the general rationale behind the thesis (Section 1.4).

1.1 Studies on human amnesic patients

A number of behavioural tasks have been developed for use with animals in an attempt to characterize brain structures that are engaged in distinct forms of learning and memory. Recognition memory is typically studied by presenting the subject with a familiar and unfamiliar stimulus, and then determining whether or not the subject can detect the difference in relative familiarity. The development of recognition tests for animals centered on descriptions from reports on human amnesic patients. Therefore, prior to describing the behavioural tasks for use with animals it is important to first describe features of the human amnesic syndrome. Indeed, traditional theories of memory largely grew from these human case studies as well as later research using nonhuman animals in attempts to model the human amnesic syndrome.

Reports of amnesia following brain damage have been documented since the late 1800s. The description of memory loss was associated with chronic alcohol abuse, and was later coined Korsakoff's syndrome. Postmortem brain tissue analysis of these patients that suffered amnesia

revealed damage surrounding the third ventricle and the mammillary bodies (Gudden, 1896; Wernicke, 1881, as cited in Andersen, Morris, Amaral, Bliss, & O'Keefe, 2007). Around the same time, Bekhterev (1900) described a similar condition of patients suffering from memory loss, who upon postmortem brain tissue analysis, were found to have bilateral damage to the medial temporal lobe (MTL) (as cited in Andersen et al., 2007). Thus, these early reports suggested that damage to the diencephalon and the MTL were associated with amnesia. These structures, albeit topographically separate, share afferent and efferent connections, however, for the purpose of this thesis I will focus exclusively on research that has examined the effects of damage to the MTL on recognition memory.

1.1.1. Medial temporal lobe (MTL) amnesia

1.1.1.1 Anatomy of the MTL

The MTL receives highly processed information from all sensory modalities via input from the association cortices. The MTL is comprised of several highly interconnected structures, namely the hippocampus (HPC), the amygdala (AMY) and the parahippocampal gryus, which consists of the entorhinal (EC), perirhinal (PRh), and parahippocampal (PH; postrhinal in the rat) cortices. Figure 1.1 illustrates the human and nonhuman primate brain depicting the location of the major structures of the MTL. Briefly, the HPC consists of the CA subfields (CA1-CA3), dentate gyrus, and subicular complex (subiculum, presubiculum, and parasubiculum). The HPC is caudal to the AMY and dorsal to the EC, PRh and PH. The HPC shares reciprocal connections with the EC, PRh, and PH (Lavenex & Amaral, 2000). The EC is medially adjacent to the PRh, and both are rostral to the PH (Lavenex & Amaral, 2000).

The PRh and PH are the first stage in the integration of information from the neocortex within the MTL. The PRh and PH receive projections from multiple unimodal and polymodal association cortices and are a major source of EC neocortical input. The PRh and PH form reciprocal connections, and also have direct afferent connections with the HPC. The EC, however, provides the majority of neocortical input to the HPC via the perforant path (Insausti, Amaral, & Cowan, 1987; Lavenex & Amaral, 2000; Suzuki & Amaral, 1994). The EC is also involved in the major output pathway that relays information back to the neocortex. Indeed, the EC has efferent projections to the PRh and PH, which project back to the same unimodal and polymodal association cortices that provided initial input to the PRh and PH (Lavenex & Amaral, 2000; Suzuki & Amaral, 1994).

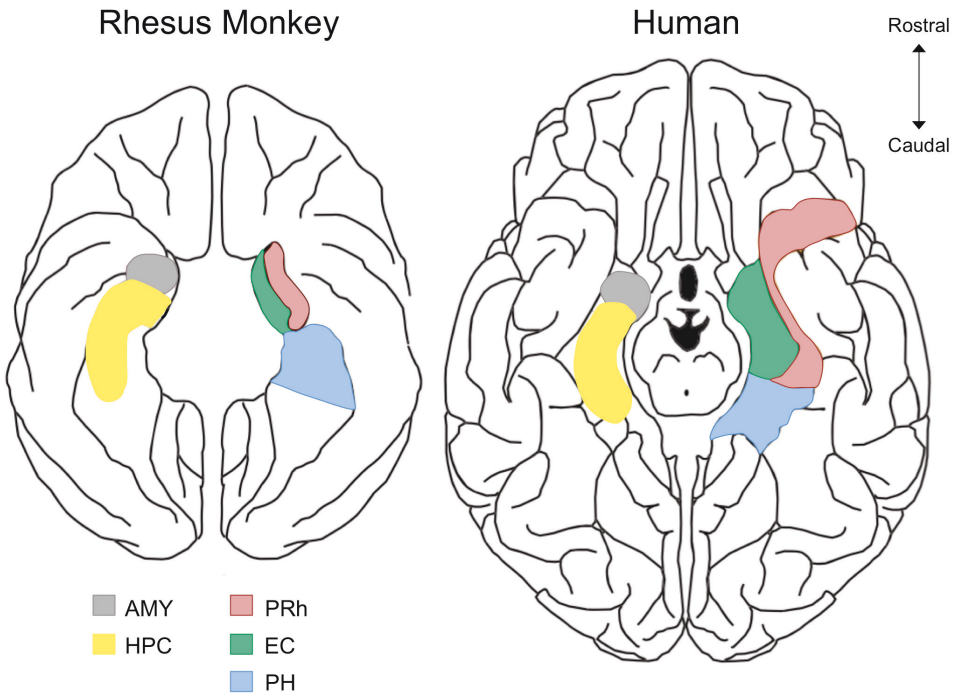

Figure 1.1. Illustrations of the ventral view of a rhesus monkey brain (left) and human brain (right) depicting the major structures in the medial temporal lobe. The image is adapted from Murray, Bussey, and Saksida (2007). Note: AMY = Amygdala; HPC = Hippocampus; PRh = Perirhinal cortex; EC = Entorhinal cortex; PH = Parahippocampal cortex.

The fimbria-fornix is the major fiber bundle that connects the HPC to the diencephalon (thalamus and hypothalamus) via both input and output projections (Amaral, Scharfman, & Lavenex, 2007). The AMY, which consists of a cluster of nuclei, receives input from both cortical and subcortical brain structures. The AMY receives projections from both unimodal and polymodal sensory cortices, and shares reciprocal projections with the HPC, EC, PRh and PH. Lastly, the AMY has substantial efferent projections to the prefrontal cortex, thalamus, and hypothalamus (Freese & Amaral, 2009).

1.1.1.2 Research from human neuropsychological patients

Although there were some findings from amnesic patients in the early 1900s implicating MTL damage with memory loss, extensive investigation into MTL damage and memory impairments was sparked by one amnesic patient in particular. In 1953, Henry Molaison (known as Patient H.M. in the scientific literature) underwent bilateral medial temporal lobectomy to treat a severe case of epilepsy (Scoville & Milner, 1957). The surgery was successful in that it greatly reduced the frequency of H.M.'s seizures. However, it resulted in an unforeseen and profound effect on H.M.'s behaviour: He had severe anterograde amnesia (an inability to form new memories) and mild retrograde amnesia (an inability to recall events that occurred prior to the surgery), however, his remote memories were intact (e.g., memories from childhood). Specifically, H.M. could not consciously recall information relating to facts and autobiographical events, a type of memory that is now referred to as *declarative* (Squire, 1986) or *explicit* memory (Graf & Schacter, 1985).[2] Moreover, H.M.'s short-term memory appeared normal, as he could retain information by rehearsing it, however, he could not hold information in memory for long periods of time, revealing deficits in long-term memory storage. Despite this impairment, his intellectual, attentional, and linguistic skills remained intact, as well as his personality (Scoville & Milner, 1957). Moreover, comprehensive neuropsychological tests further revealed that some forms of learning and memory were preserved, such as motor skill learning (*procedural* memory), classical conditioning, and perceptual priming (Cohen & Squire, 1980; Milner, Corkin, & Teuber, 1968; Warrington & Weiskrantz, 1968, 1974). Interestingly, H.M. had no conscious recollection of learning or performing these aforementioned learning and memory tests despite having performed them numerous times (Cohen & Squire, 1980; Milner, 2005). Thus, memory

[2] The descriptive term *declarative* refers to a "type" of memory, whereas *explicit* relates to the demands of the particular task (i.e., whether it requires intentional or conscious recollection).

for information that did not require deliberate recollection, or declarative knowledge, appeared to be spared. These types of memory are referred to as *nondeclarative* (Squire, 1992) or *implicit* (Graf & Schacter, 1985) memory. The neuropsychological testing conducted on patient H.M. and other patients with MTL damage revealed that there were multiple dissociable memory systems.

The extent of H.M.'s MTL lesion was based on Scoville's post-operative drawings, which were only speculative at the time due to the lack of neuroimaging techniques to evaluate postoperative outcomes. Scoville intended to perform a bilateral resection that would extend 8 cm from the rostral tips of the temporal lobes, with the intent to remove the uncus, AMY, HPC, and PH (Scoville & Milner, 1957). In addition to patient H.M., Scoville and Milner's neuropsychological report described several other patients that had undergone varying degrees of bilateral resections of the temporal lobes. Similar to H.M., the patients that had suffered memory loss also sustained damage to the HPC, and the extent of HPC damage appeared to be correlated with the severity of the memory impairments. Consequently, it was concluded that the memory loss was associated with HPC damage (Milner et al., 1968; Penfield & Milner, 1958; Scoville & Milner, 1957). Over the next few decades, the focus was on the HPC as the major anatomical structure underlying MTL amnesia. In the early 1990s following improvements in *in vivo* biological research methods, magnetic resonance imaging (MRI) scans revealed that approximately half of patient H.M.'s HPC remained intact, while other MTL structures such as the PRh, EC, PH, and AMY received extensive damage (Corkin, Amaral, Gonzalez, Johnson, & Hyman, 1997). This was an important finding because it suggested that H.M.'s impairments, which included visual recognition, were not entirely the result of HPC damage. The resolution of the MRI scans at the time, however, were not high enough to provide detailed anatomical boundaries of the MTL lesion, thus only following the death of H.M. in 2008 was there conclusive information on the extent of the lesion (Annese et al., 2014). The postmortem imaging in combination with direct histological analyses of H.M.'s brain revealed that the resection only extended ~5 cm, not 8 cm, posterior to the temporal tips, such that portions of the HPC remained intact, whereas the majority of the PRh and AMY were removed, and the entirety of the EC was removed (Annese et al., 2014; Augustinack et al., 2014).

1.1.2 Limitations of research on human neuropsychological patients

There are certain limitations to studying recognition memory in human patients that have sustained damage to the brain. As briefly detailed above, it is difficult to conclude which brain

structure contributes to specific memory impairments. Moreover, damage is typically not restricted to anatomically circumscribed areas of the brain and in cases where it is, rarely is the damage complete. In addition to the attributes of the lesion, there is a lack of information regarding the patient's memory abilities prior to the brain damage. This piece of information is crucial when determining whether or not the brain damage is *responsible* for subsequent impairments. Indeed, simply administering a memory test after a person has incurred brain damage can only provide information on whether or not an ability is impaired, not if it is responsible for that impairment. Consequently, nonhuman animal models provide the opportunity for systematic investigation of these questions. In animal studies, selective lesions can be made to determine whether or not specific functions remain intact and tests can be administered prior to the surgery.

1.2. Development of nonhuman primate models of MTL-damaged-produced amnesia

When researchers first studied patients with MTL-damaged-produced-amnesia in the 1950s and 1960s, very little was understood about memory processes and the type of memory tasks that could be used to model MTL amnesia, as the type of learning and memory abilities that were impaired and spared following damage to MTL structures were not fully characterized. Consequently, researchers had difficulty developing appropriate behavioural tasks for animals that were analogous to those failed by human amnesic patients, and the first behavioural tests created to model brain-damaged-produced-amnesia in nonhuman animals failed (Duva, Kornecook, & Pinel, 1999). As studies on human amnesic patients in laboratories shed light on the spared and impaired memory abilities following MTL damage, it advanced the development of behavioural tests for nonhuman animals analogous to those failed by human amnesic patients.

In terms of deciding which MTL structures to lesion in order to model human amnesic syndrome, there was a focus on HPC lesions primarily because findings from humans suggested there was a correlation between the extent of presumed HPC loss and severity of memory impairments (Duva et al., 1999; Milner et al., 1968; Scoville & Milner, 1957). Also, Scoville and Milner (1957) observed that patients who sustained bilateral damage to other MTL structures, such as the AMY, in the absence of HPC damage, did not suffer from amnesia.

By the late 1970s researchers produced the first successful model of MTL amnesia using nonhuman primates, and a pivotal factor in this breakthrough was the development of one particular behavioural task: the *trial-unique delayed nonmatching-to-sample* (DNMS) task

(Gaffan, 1974; Mishkin, 1978; Mishkin & Delacour, 1975). Using the DNMS task, Mishkin (1978) showed that MTL lesions impaired nonhuman primates' performance on the test. Following this discovery, which will be elaborated in the next section, the DNMS task became the most widely used test to assess MTL-damage-produced amnesia in animals. The following sections describe the task and the subsequent research examining the effects of MTL lesions on nonhuman primates' performance on the DNMS task.

1.2.1. The trial-unique delayed nonmatching-to-sample task

The trial-unique DNMS task (henceforth simply referred to as DNMS) requires the subject to make a judgment of familiarity about a recently presented object. A trial on the DNMS task consists of two phases: a sample and a choice phase. On the sample phase, an object (called the sample) is briefly presented to the subject by placing it over a food well and allowing the subject to displace it to retrieve a food reward. The sample is removed from view for a brief retention interval (e.g., 5 s), and is then presented again alongside a novel (unfamiliar) object. The subject receives a reward if it selects the novel object in the pair. Different sample and novel objects are used on each trial, so reliably accurate performance requires that the subject can recognize the sample objects. Several trials are conducted within a session, and a performance criterion (e.g., 90 correct choices in 100 trials) is implemented to confidently determine that the subject has mastered the nonmatching rule. Thus, the main dependent measure is the accuracy of responding. Nonhuman primates quickly learn the reward contingency with high accuracy (>90%) when the delay lasts only a few seconds. After reaching the performance criterion, the demands of the task are made increasingly difficult by either increasing the length of the retention interval or by increasing the number of items in a list to be remembered (e.g., 2, 3, 4…).

The development of the DNMS task was significant because it was analogous to the recognition tests used with human amnesic patients. For example, on human visual recognition tests, the participant is presented with a list of items (e.g., words or pictures), and then the list is taken away. After a retention interval, the participant is presented with a list that contains a combination of previously presented items and new items. The participant is asked to identify the items that appeared on the original list (MacDougall, 1904). Correspondingly, the DNMS task requires that the subject distinguish between an object that was previously presented (familiar) and a new one (unfamiliar).

The DNMS is considered to assess *working-memory*—memory for information that is only needed for one trial on a task (Honig, 1985). There were several key factors that made the DNMS task successful compared to the nonspatial working-memory tasks devised for nonhuman primates in the 1950s and 1960s. The first feature was the use of a large set of stimulus objects, rather than a small set of recurring objects. The nonspatial delayed-response paradigms in the 1950s and 1960s used only a small set of stimuli across trials, resulting in the stimuli becoming familiar after only a few trials (Correll & Scoville, 1965; Etkin & D'Amato, 1969; Mishkin & Weiskrantz, 1958; Scheckel, 1965; Weinstein, 1941). Thus, on each trial, the nonhuman primate was required to remember which of two equally familiar stimuli it had encountered more recently. Consequently, this makes the task a measure of *recency memory* (memory for the temporal order of stimuli), not recognition memory, which nonhuman primates have difficulty successfully performing when the delay lasts only a few seconds. A second feature was the introduction of longer retention intervals, ones that lasted more than several seconds. The retention intervals used on the existing tasks were not sufficiently long to observe whether or not an impairment existed. The longest delay used was ~10 s, which is within the limits of short-term memory, and is largely spared in human amnesic patients (Correll & Scoville, 1965; Mishkin, 1954; Mishkin & Pribram, 1954). Thus, the nonhuman primates with MTL lesions in those studies may have been impaired on the task, but it went undetected because only short delays were used. This was later confirmed, when research revealed patients with brain-damaged produced amnesia can successfully perform delay response tasks following 10-s delays (Sidman, Stoddard, & Mohr, 1968). This was further confirmed when Zola-Morgan and Squire (1985) found that nonhuman primates with bilateral lesions to the HPC and AMY performed similar to controls on delayed response tasks when the delay was 8 s, but their performance significantly declined when the delay was increased to 15 or 30 s.

In 1978, Mishkin published research on the first animal model of human amnesia using trial-unique stimuli on a DNMS task. Nonhuman primates with combined lesions to the HPC and AMY, but not separate lesions to each structure, were severely impaired on the test revealing a delay-dependent impairment. They were able to reach a performance criterion when the delay between the sample and test phase was short (10 s) but they were impaired relative to controls when the delay was increased to 30, 60, and 120 s (Mishkin, 1978). The accurate performance at short delays indicated that the nonhuman primates were capable of executing the rule, thus, the

deficit caused by the combined HPC and AMY lesion following longer delays was unlikely to be due to a failure to remember and apply the nonmatching rule. Instead, the results suggested that combined HPC and AMY lesions had no effect on short-term memory, but they prevented the formation of memories if the delay exceeded 30 s. The results from this study, along with those from other experiments at the time, led to the conclusion that the HPC and AMY equally contributed to the type of memory loss observed in human MTL amnesia (Mishkin & Murray, 1994; Zola-Morgan & Squire, 1985). Moreover, these findings were convincing because the observed delay-dependent impairment on the test was similar to those observed in human patients who had suffered from MTL damage –performance was normal on visual recognition tests when the retention interval was a few seconds, but declined when the retention interval was increased to several minutes (Squire & Zola-Morgan, 1991; Zola-Morgan & Squire, 1985). Accordingly, the object-recognition task developed for nonhuman primates appeared to assess similar underlying memory processes affected by temporal lobe damage while sparing memory for procedural learning, which was not affected by damage to the MTL (Squire & Zola-Morgan, 1991). This was later supported with research showing that human amnesic patients with bilateral MTL damage had similar performance impairments on the DNMS task used for nonhuman primates –their performance was normal following a 5-s delay but declined as the delay was increased to 15 and 60 s (Squire, Zola-Morgan, & Chen, 1988). Thus, testing nonhuman primates with MTL damage on object-recognition tasks provided a successful model of human MTL amnesia.

1.2.2. Elucidating the role of specific MTL structures in amnesia

Following the successful development of an animal model of MTL amnesia, there was still emphasis on the HPC as the primary structure associated with stimulus recognition impairments. Some researchers found evidence to support this hypothesis by showing that HPC lesions alone produced DNMS impairments in nonhuman primates following long retention intervals (Mahut, Zola-Morgan, & Moss, 1982; Zola-Morgan & Squire, 1986). Others, however, reported only mild deficits following HPC lesions (Mishkin, 1978; Mishkin & Murray, 1994). One variable that seemed to differ between these studies was the extent of presurgery training the subjects received. Nonhuman primates with extensive presugery training were only mildly impaired on the task, whereas those with no presurgery training were impaired on the task (Mahut et al., 1982; Zola-Morgan & Squire, 1986). This factor does seem to influence whether

or not an impairment will be observed, suggesting HPC lesions disrupt other skills contributing to normal performance on the task, such as learning the nonmatching rule and withholding hasty responses (Mumby, 2001).

A more crucial discovery based on research conducted over the next decade revealed that the observed impairments in nonhuman primates on the DNMS task following combined lesions made to the HPC and AMY were the result of incidental damage made to the rhinal cortex (i.e., the EC and PRh) during the surgical removal of the HPC and AMY (Duva et al., 1999). At the time, it was difficult to perform HPC or AMY lesions without causing damage to the nearby cortex, and the role of the rhinal cortices in memory were not fully considered. Studies began to reveal that select rhinal cortex lesions, specifically the PRh, impaired both the acquisition of, and performance on, the DNMS task (Meunier, Bachevalier, Mishkin, & Murray, 1993; Mishkin & Murray, 1994; Murray & Mishkin, 1986; Suzuki & Amaral, 1994; Suzuki, Zola-Morgan, Squire, & Amaral, 1993; Zola-Morgan, Squire, Amaral, & Suzuki, 1989; Zola-Morgan, Squire, Clower, & Rempel, 1993). Conversely, select lesions made to the HPC and AMY without damaging cortical tissue did not result in impairments (Zola-Morgan, Squire, Amaral, et al., 1989). Thus, overtime it was becoming increasingly clear that the HPC was not critical for normal performance on the DNMS task, whereas the PRh appeared to play a critical role in the ability to recognize previously encountered stimuli.

Since the discovery of MTL damaged-produced-amnesia, decades of neuropsychological research, including converging evidence from animal studies, have revealed that there are different "types" of memory, and in addition to the MTL supporting the formation of memories, there is functional diversity within MTL structures—each with specialized functions that appeared to subserve distinct mnemonic processes. To illustrate, research has implicated the HPC in playing an integral role in processing allocentric spatial and contextual information, whereas the PRh appears to be critical for object-recognition memory (Aggleton & Brown, 1999; Brown & Aggleton, 2001; Murray & Richmond, 2001; O'Keefe & Nadel, 1978). Evidently, over time the emphasis on developing animal models of MTL amnesia evolved to focus on elucidating the functional role of distinct MTL structures in specific learning and memory processes.

1.3 Object-recognition paradigms for use with rats

While research was being conducted on nonhuman primates, attempts were being made to develop nonspatial memory tests for use with rats. Using rat models provides the opportunity for large-scale experiments, as nonhuman primate experiments are costly, which consequently limits both the number of experiments that can be conducted and the sample size within experiments. To date, rodents are the most widely used animals in experiments assessing the neural basis of object-recognition memory. The following section describes the two most common object-recognition paradigms developed for use with rats, and the findings from experiments that involved discrete lesions made to MTL structures. Though the two tasks are used to measure the same underlying ability, they are quite different from one another and provide qualitatively different information regarding the status of object-recognition. A description of the drawbacks associated with each task will be further elaborated to make clear the rationale for developing a new test of object-recognition for rats.

1.3.1 DNMS paradigms

Beginning in the mid 1980s, researchers examined object-recognition memory in laboratory rats using variants of the trial-unique DNMS task (Aggleton, 1985; Mumby, Pinel, & Wood, 1990; Rothblat & Hayes, 1987). The development of these rodent DNMS paradigms advanced our understanding of the contributions of distinct MTL structures to object-recognition memory by providing the opportunity for large-scale experiments. They also helped bridge the findings in nonhuman primates and humans. Similar to DNMS procedures used for nonhuman primates, the rodent versions also consist of a sample phase, retention interval, and a choice phase. Although there were a few DNMS task variants, they all assess performance based on the same two dependent measures: 1) the mean number of trials needed to reach a performance criterion and 2) the mean percent correct choices on the task.

The first rodent DNMS task was developed by Aggleton (1985), and was conducted using a Y-maze apparatus (see Figure 1.2). The three arms of the Y-maze apparatus were separated by guillotine-like doors placed in the center of the maze. On a trial, one arm was

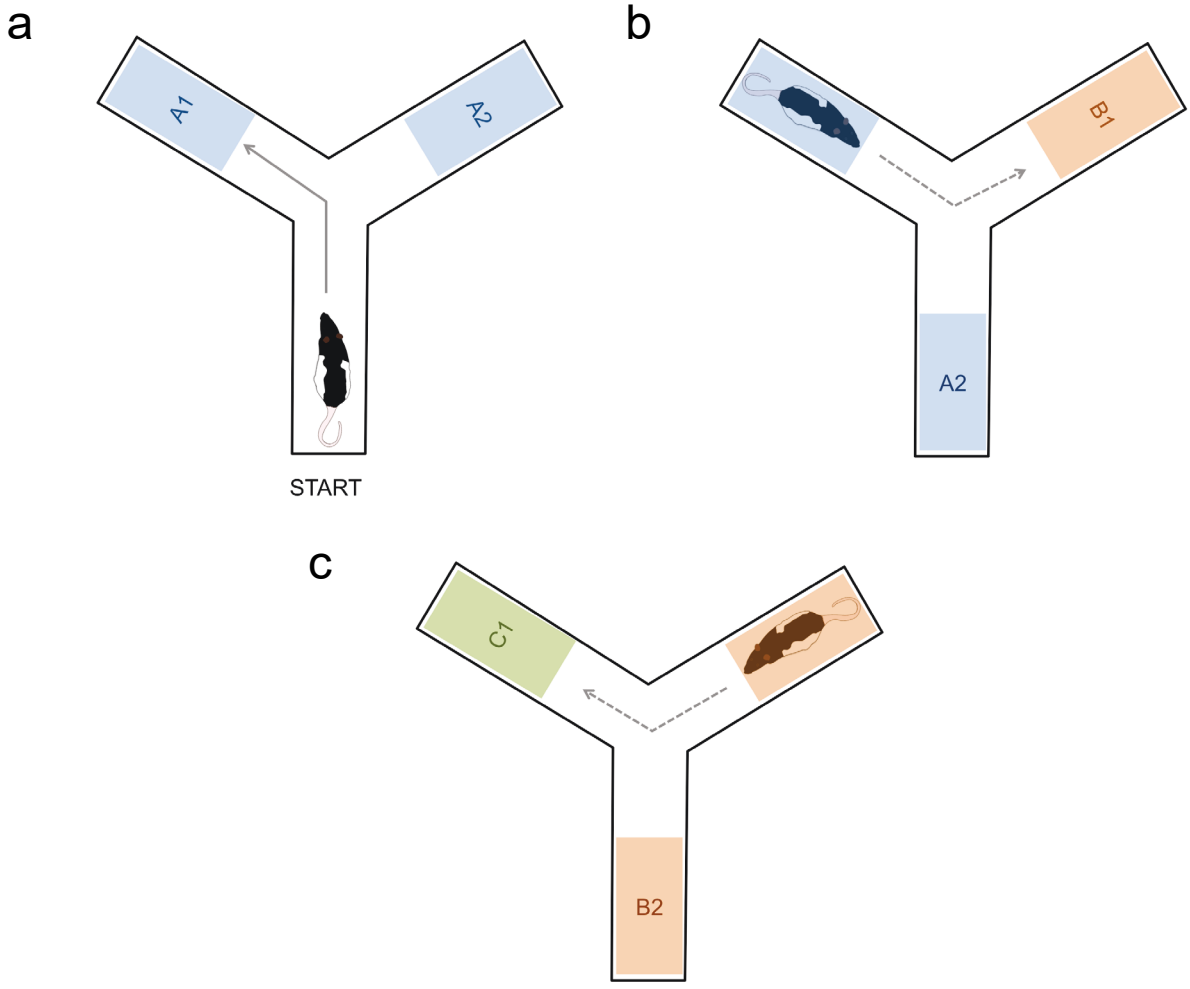

Figure 1.2. Aggleton's Y-maze DNMS task paradigm. The top two figures depict Trial 1 (a) the beginning of Trial 1 and (b) the first choice. The bottom figure (c) depicts the start of Trial 2. The coloured portions of the Y-maze arms represent the removable goal boxes (stimulus objects). The dashed grey line represents the correct choice. Image adpated from Aggleton (1985).

designated the start box, while the other two were designated goal boxes. The stimulus objects consisted of 40-50 different pairs of goal boxes, which could be attached to the ends of the arms. These boxes differed in their visual and tactile properties, such that they were painted in different colours and patterns, and had different floor substrates (e.g., sandpaper, metal, or cloth). Each pair of boxes contained an identical object fastened to the floor. A hole at the back of each goalbox made it possible to deliver a food reward to the rat after it had made its choice. A trial on the task began by placing the rat in the start box and raising the guillotine-like doors to expose two identical 'sample goal boxes' (e.g. A1 and A2). The rat was rewarded with food pellets for entering either one (e.g., A1). Then, the rat was enclosed in this sample goal box for 20 s while the experimenter replaced the start box with either the copy of the sample goal box (A2) or a novel goal box (B1). In the latter case, the experimenter simply left the sample goal box (A2) in its previous location. At the end of the 20 s, the guillotine door opened and the rat made a choice between entering the sample or novel goal box. The rat was rewarded if it chose the novel goal box (B1). This goal box then served as the sample goal box on the subsequent trial, and the other two goal boxes were randomly replaced with a copy of the now-sample goal box (B2) and a novel goal box (C1). The trials proceeded in this sequential fashion. To implement longer retention delays, after the rat was confined to the goal box for 20s, the box was removed (with the rat in it) and replaced with a featureless box, which the rat was then "tipped" into, where it remained for the designated delay period (Aggleton, Hunt, & Rawlins, 1986, p. 136). At the end of the delay, the guillotine door was raised and the rat could make a choice. On trials where a rat made an incorrect choice, it was provided the opportunity to make a second choice. This was a necessary feature of the paradigm because the rat had to enter the goal box that would serve as the sample for the subsequent trial (Aggleton et al., 1986).

Rats received 10 trials per day on the task and were trained using a 0-s delay until they reached a performance criterion of at least 80% correct choices on five consecutive days (40 correct choices out of 50). Aggleton reported that rats required a mean number of 130 trials to reach the performance criterion (excluding criterion trials). After reaching criterion, rats were tested using a 20, 60, and 120-s delay and were found to maintain good performance: 84%, 74%, and 80%, respectively (Aggleton, 1985). Importantly, this study was the first to demonstrate that rats, like nonhuman primates, could perform well on a DNMS task.

The features of Aggleton's DNMS paradigm, however, differed from nonhuman primate DNMS tasks in several ways, and this was hypothesized to potentially change the cognitive demands on the task, consequently making it more difficult to generalize findings from rats to nonhuman primates (Mumby et al., 1990). First, the stimulus objects were quite different from those used on the nonhuman primate DNMS version. In the nonhuman primate version, the stimuli consisted of "junk" objects in which the subject physically manipulated by displacing it from over a food well, not an object that was fixed to the floor. Moreover, the goal boxes in which these objects appeared incorporated varied spatial features that could serve as a cue to distinguish between stimuli. This feature makes the task not necessarily a discrete test of nonspatial information, like those used with nonhuman primates. Additionally, the disruption during the delay that came with changing the goal boxes and tipping the rat into a different box was likely distracting to the rats. Second, unlike conventional procedures used to test nonhuman primates, trials on Aggleton's DNMS task proceeded sequentially such that the novel goal box on the first trial became the sample goal box on the second trial. In nonhuman primate DNMS, two new objects serve as the sample and novel on each trial. Lastly, the number of stimuli used was quite small compared to nonhuman primate DNMS tasks that use 200+ objects. As stated above, using small numbers of stimuli can potentially lead to the recognition task becoming a test of recency memory, which is much more difficult for animals to solve.

At the same time, Rothblat and Hayes (1987) developed a rodent-based DNMS task that more closely matched the tasks used for nonhuman primates (see Figure 1.3). Namely, this consisted of the use of discrete trials and a large pool of 250 junk objects that could be positioned over food wells in which a rat could easily displace to retrieve a food reward. The apparatus consisted of an elevated rectangle-shaped platform with 2 cm high walls. There was a start area at one end of the platform and three recessed food wells at the other, which were connected to a food dispenser operated by the experimenter. At the beginning of a trial, the rat was retained in the start area by a guillotine door that the experimenter controlled. Once the door was raised, the rat could run down the platform and displace a single object (sample object) placed over the middle food well and retrieve a food reward. Afterwards, the experimenter picked up the rat and returned it to the start area where it remained throughout the retention interval. Following the delay, the door was opened and the rat was presented with the sample object and a novel object,

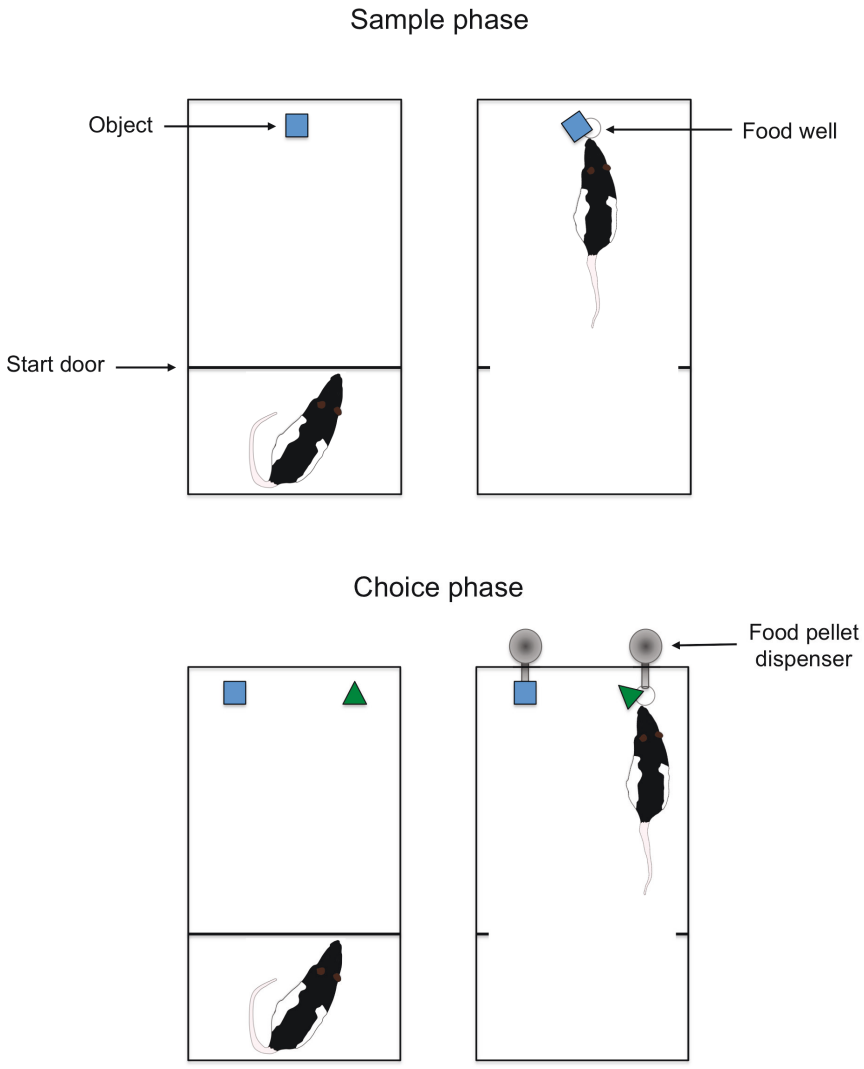

Figure 1.3. A schematic of one trial of the DNMS paradigm developed by Rothblat and Hayes (1987) for testing rats. The top image depicts the sample phase, whereby a single sample object (blue square) is placed over one food well. The start door is raised and the rat is rewarded for displacing the sample. The bottom image depicts the choice phase. The sample and a novel object (green triangle) are each placed over a food well; once the start door is raised the rat is rewarded if it displaces the novel object. Image adapted from Rothblat and Hayes (1987).

each over one of the lateral food wells. If the rat displaced the novel object first, it received a food reward. Rothblat and Hayes gave rats 12 trials per day using a 10-s delay. The rats were trained to reach a performance criterion of at least 75% correct choices on three consecutive days (27 correct choices out of 36). Rats reached a mean accuracy of 77% following an average of 178 trials. When the delay was increased to 30 and 120 s, accuracy decreased to 70% and 63%, respectively (Rothblat & Hayes, 1987). Similar to nonhuman primates, there was a delay-dependent decrease, despite the rats' accuracy scores at respective delays being much lower.

Although Rothblat and Hayes's DNMS task more closely resembled the nonhuman primate DNMS, there were still drawbacks associated with it. Specifically, it appeared more difficult for rats to master, as they only reached a mean accuracy score of 77% following 178 training trials, and their scores at the respective delays were much lower compared to scores obtained by nonhuman primates. Two potential factors that may have contributed to this diminished performance are the retention interval used during training, and handling the rat between the sample and choice phase. Compared to Aggleton's DNMS version, the delay imposed between the sample and choice phase during task acquisition was rather long (10 s), and this may have made it more difficult for rats to remember the sample object. Additionally, handling the rat between the sample and test phase may have acted as a form of distraction during the retention interval—a factor shown to disrupt performance on DNMS tasks (Zola-Morgan, Squire, & Amaral, 1989; Zola-Morgan & Squire, 1985). Another problem associated with Rothblat and Hayes' version was the rather lenient performance criterion of at least 75% correct choices across three consecutive days. Implementing a high performance criterion is essential on DNMS tasks in order to confidently determine that a rat has mastered the reward contingency. Moreover, using a stringent criterion allows for the ability to detect even slight impairments in performance following some form of treatment (e.g., surgical lesion) (Mumby, personal communication, July 2012).

Mumby and colleagues hypothesized that these aforementioned drawbacks limited a rat's full potential performance on a DNMS task (Mumby, Pinel, & Wood, 1990). Accordingly, to achieve more comparable levels of accuracy with nonhuman primates on DNMS tasks using rats, Mumby and colleagues developed a new DNMS paradigm, one that addressed the limitations of the previous ones. This was achieved by incorporating the following features: 1) a strict performance criterion, 2) a short retention interval during task acquisition, 3) a large pool of junk

objects in which the rat would physically manipulate, and 4) doors to control the rat's access to the sample and test phase, removing the need to handle the rat between trials. These changes required a modification to the training procedures and the apparatus design, which I will briefly describe next.

The apparatus consisted of an elevated rectangular-shaped platform enclosed by tall (40 cm) walls (see Figure 1.4). There were two recessed food wells located at each end of the platform, which were each connected to a long funnel that allowed the experimenter to deliver food pellets. An opening next to the food wells provided the experimenter with access to place objects over the food wells and to remove them. Two guillotine-like doors were located in the middle of the platform. The purpose of the doors was to allow the experimenter to control the rat's access to different parts of the apparatus by manually raising and lowering them. Importantly, implementing the use of doors removed the need for the experimenter to handle the rat within and between trials. Similar to previous DNMS tasks, the experimenter plays an active role, as he/she is standing directly next to the apparatus administering each trial. A trial begins with the rat confined to the center of the apparatus and the experimenter raises one door. The rat walks down the runway and is briefly presented with an object (the sample). It displaces the object and receives a food reward. The rat then returns to the center area where it is given a retention interval that can last from several seconds to minutes. During this time, the experimenter places the sample next to a novel object at the opposing end of the apparatus. After the retention interval, the opposite door is raised and the rat is presented with a choice. If it displaces the novel object, it receives a reward. Different object are used on each trial, selected from a pool of 350 objects.

Rats were trained on the task using a 4-s delay (the shortest interval that could be implemented as the experimenter set up the objects for the trial). Rats were trained to reach a performance criterion of at least 21 out of 25 correct choices on two consecutive sessions (at least 84% correct choices). After task acquisition using a short delay, training continued using longer retention intervals lasting seconds to minutes. The rats were trained on these longer delays until they re-attained the performance criterion, or reached a maximum number of 200 trials. Mumby and colleagues revealed that rats reached a mean accuracy of 90% following an average number of 235 trials at the shortest delay. Although performance dropped to 81% when the delay was increased to 60 s and then to 77% when increased to 120 s, performance remained

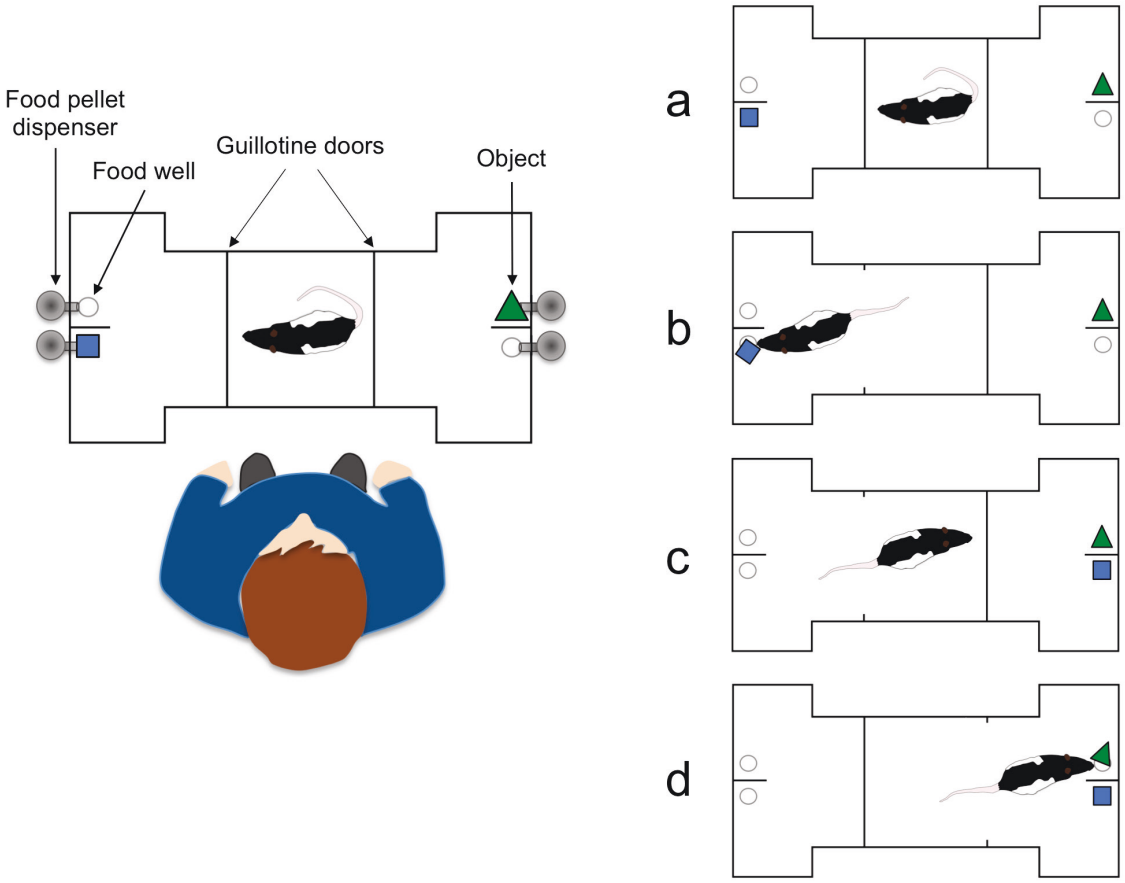

Figure 1.4. Illustration of the rodent DNMS paradigm developed by Mumby, Pinel, and Wood (1990). The left schematic depicts the location of the experimenter relative to the apparatus and the right schematic depicts the stages of one trial on the task: (a) The trial begins with the rat enclosed in the center with the two guillotine doors closed and the objects for the trial are setup at opposite ends (blue square and green triangle). (b) The guillotine door is raised and the rat approaches and displaces the sample object (blue square) and receives a reward; (c) the retention interval during which the experimenter places the sample next to a novel object (green triangle); and (d) the test whereby a rat makes a choice—if it displaces the novel, it receives a reward.

significantly above chance (Mumby et al., 1990). Notably, these accuracy scores were comparable to those achieved by nonhuman primates, and this DNMS paradigm most closely resembled the nonhuman primate version. It thus provided a suitable means to generalize findings from rats to nonhuman primates. Indeed, the paradigm developed by Mumby and colleagues has been used in the majority of published reports assessing DNMS performance in rats.

1.3.1.2 Examining the effects of MTL lesions on rats' DNMS performance

There is anatomical and functional similarity between the brains of rodents and nonhuman primates. Rodents have brain structures that are homologous to parts of the MTL in primates (see Figure 1.5). The question remains, however, to what extent object-recognition memory involves similar cognitive processes in rodents and primates. Findings from experiments assessing DNMS task performance in rats following MTL lesions demonstrate that the task can be successfully used to detect impairments in object-recognition memory in rats. As will be outlined in the following section, research on rats appears to support the theories of MTL function developed from studies using nonhuman primates.

The first lesion experiments in the mid 1980s focused on the HPC because at the time this structure was still hypothesized to play a critical role in object-recognition memory. Aggleton, Hunt, and Rawlins (1986) were the first to develop a rat model of MTL amnesia using their Y-maze apparatus. In the study, rats received HPC lesions and were trained on the DNMS task. The results showed that compared to control rats, rats with HPC lesions successfully acquired the task at the same rate and reached the same level of accuracy following the maximum delay of 60 s. Comparatively, Rothblat and Kromer (1991) reported that lesions made to the fornix[3] after training rats on the DNMS task did not disrupt performance following a 30-s delay. Moreover, Mumby, Wood, and Pinel (1992) showed that rats with either separate or combined lesions of the HPC and AMY performed comparably to controls on the DNMS task at delays lasting up to 120 s, and only showed a slight decline in performance following a 600-s delay. Thus, consistent with nonhuman primate studies, HPC lesions in rats did not appear to disrupt DNMS

[3] The fornix is the major efferent fiber from the HPC to subcortical structures and is considered to be integral for normal HPC functioning (Aggleton & Brown, 1999). Fornix transections were considered a form of HPC lesion that had the advantage of not damaging other structures outside the HPC—one inadvertent consequence of the aspiration technique that was used at the time (Gaffan, 1972).

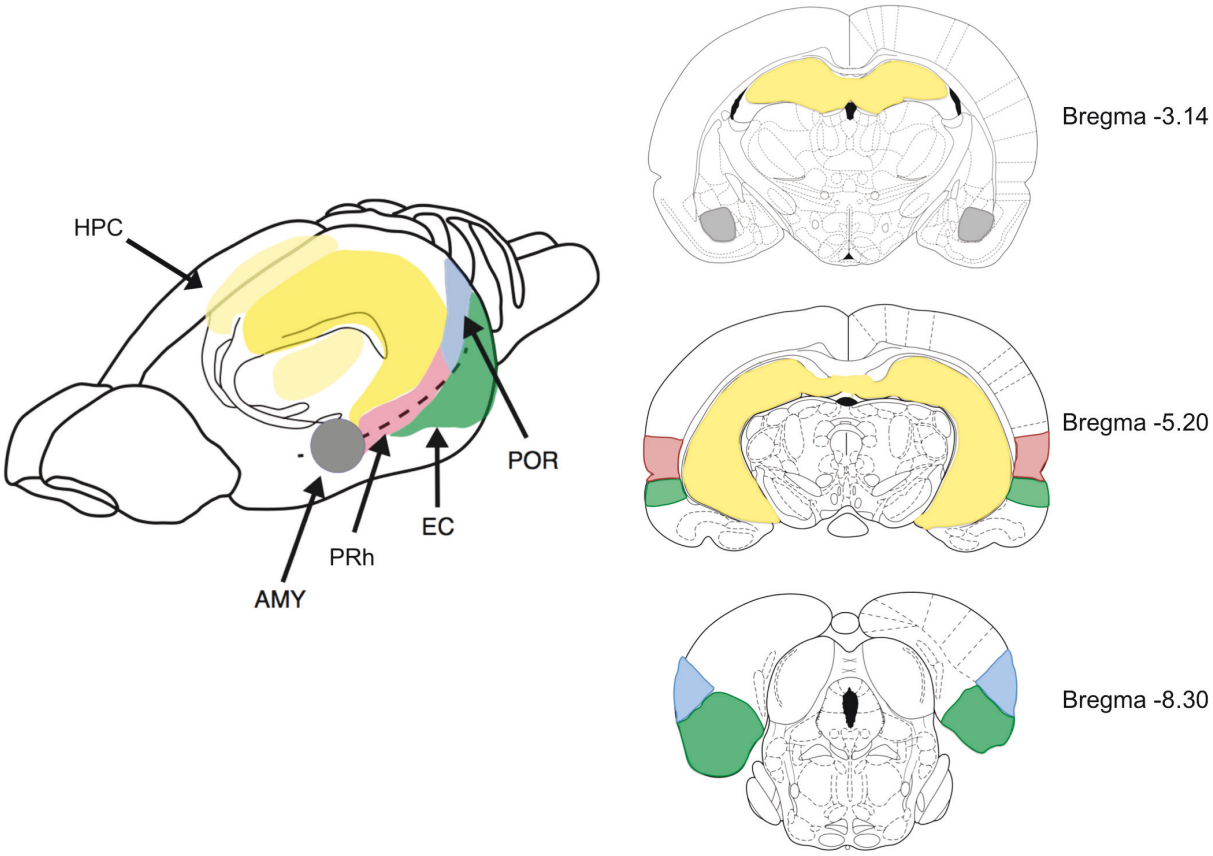

Figure 1.5. Illustration of a rat brain (left) and coronal sections (right) depicting the major structures in the medial temporal lobe. Left image adapted from Kerr, Agster, Furtak, and Burwell (2007) and right images adapted from Paxinos and Watson (1998). Note: HPC = Hippocampus; AMY = Amygdala; PRh = Perirhinal cortex; EC = Entorhinal cortex; POR = Postrhinal cortex.

performance. Conversely, one study that used the same apparatus as Mumby and colleagues observed DNMS deficits in rats with HPC damage following delays lasting 60-s or longer, but not following shorter delays (Clark, West, Zola, & Squire, 2001). However, unlike previous experiments, the rats in the Clark et al. study did not receive any DNMS training prior to HPC lesions. Interestingly, it appeared that presurgery DNMS training influenced whether or not a performance deficit was observed –a similar finding in nonhuman primate DNMS studies. Indeed, providing extensive training using different delays prior to surgery seemingly helped rats master other skills (e.g., learning to avoid distraction during the delay) that are required for good performance at longer delays that may otherwise mask normal object-recognition abilities.

Overall, findings from rodent DNMS studies were consistent with those being reported from nonhuman primates, revealing that damage to either the HPC or AMY did not produce DNMS deficits (Aggleton et al., 1986; Duva et al., 1997; Jackson-Smith, Kesner, & Chiba, 1993; Kesner, Bolland, & Dakis, 1993; Mumby, Pinel, Kornecook, Shen, & Redila, 1995; Mumby et al., 1996, 1992; Rothblat & Kromer, 1991; Shaw & Aggleton, 1993; Steele & Rawlins, 1993; Yee & Rawlins, 1994). Notably, the rodent DNMS studies helped elucidate the MTL structures that were critical for producing impairments on the DNMS task. This is because creating circumscribed lesions to the HPC without damaging surrounding rhinal cortices was much easier to do in rats than it was in nonhuman primates, due to the differences in the anatomical layout of MTL structures between the two species (Duva et al., 1999).

Only a handful of studies have been published examining the effects of PRh damage on DNMS performance in rats. The first study was by Mumby and Pinel (1994), who trained rats on the DNMS task and then tested them using several delays ranging from 4 to 600 s. Afterwards, rats received rhinal cortex (EC and PRh) lesions and were tested again at the same delays. Rats' scores on the 4-s delay was comparable to their pre-surgery scores, whereas their scores on delays of 15 s or longer had significantly declined (Mumby & Pinel, 1994). Wiig and Bilkey (1995) used the same DNMS paradigm, however, they administered PRh lesions or sham surgery prior to training rats on the DNMS task, and then assessed their performance on the task using delays ranging from 4 to 120 s. Compared to the sham-surgery rats, rats with PRh lesions required on average more trials to reach the performance criterion (but this was not statistically significant), and they were impaired on the task following delays lasting 30 s or longer (Wiig & Bilkey, 1995). The observed delay-dependent impairments in rats following PRh lesions were

consistent with findings from nonhuman primates, further supporting a critical role for the PRh, not the HPC, in object-recognition memory.

1.3.1.3 Drawbacks of the existing DNMS paradigms for rats

The rodent DNMS task developed by Mumby and colleagues appeared to provide a relatively precise measure of a rat's object-recognition abilities, and it was a useful tool to assess the effects of treatments on object-recognition memory. However, there were certain drawbacks associated with it, which resulted in most researchers abandoning it. A required feature of the DNMS task is a strict performance criterion (e.g., 85% correct choices on two consecutive sessions, or 34 correct choices out of 40). These higher performance criteria are essential to confidently determine that a rat has mastered the nonmatching rule and to detect even slight performance deficits due to some treatment. Predictably, these performance levels are achieved only through extensive and time-consuming training. Most rats require several weeks of daily training, and hundreds of trials, to reach peak performance. The mean number of trials required for rats to reach the performance criterion on the different versions of DNMS paradigms is ~285 trials (range 130-420). After the rat has acquired the task, it is typically trained using gradually longer retention intervals, which can take hundreds more trials. After training, rats receive additional sessions in which all the delays are presented in a mixed fashion. Thus, many investigators would consider this time requirement to be prohibitively long.

Moreover, even at peak performance, rats do not perform accurately when the retention interval is more than a few minutes, and for this reason most previous studies have used maximum retention delays of 120-300 s. For example, the handful of studies that assessed normal rats' performance following delays lasting more than a few minutes showed accuracy scores drop from an average of 89% using a 15-s delay to an average 59% following a 10-min delay (Mumby & Pinel, 1994; Mumby et al., 1990, 1992). Thus, conventional DNMS tasks cannot be used to study long-term memory with retention intervals of several minutes, or hours, or days.

Lastly, DNMS tasks are difficult to administer and require experienced experimenters. The experimenter is in the room with the rat and plays an active and ongoing role in administering the trials (Herremans, Hijzen, & Slangen, 1995; Mumby, 1995; Mumby, Kornecook, Wood, & Pinel, 1995). So close to the test subjects, the experimenter must be mindful of making any movements and sounds that could distract the animal. Also, an

experimenter could unknowingly deliver cues to the rat as to which object will be rewarded on the choice test by, for example, making a slight body movement in anticipation of the rat making a correct choice on the test or leaving odour cues on the objects as a result of touching them between the sample and choice phase (Mumby, 2005, p. 385). Thus, due to the challenges faced employing the DNMS task for rats, researchers have generally abandoned it in favour of the *novel-object-preference* (NOP) test.

1.3.2 The novel-object-preference (NOP) test

The NOP test leverages a rat's natural tendency to investigate novel objects more than familiar ones when both are presented in a familiar environment (Berlyne, 1950; Besheer & Bevins, 2000; Ennaceur & Delacour, 1988). Thus, this task does not require extensive periods of training. Conventional NOP procedures vary slightly from one laboratory to another, but all are generally similar (see Figure 1.6). At the beginning of a trial a rat is placed in an arena, where it is allowed to explore and investigate two identical sample objects for a designated amount of time (3 or 5 minutes) or until it accumulates a set total amount of time investigating the sample objects (e.g., 30 s). The latter method ensures that all rats spend equal time investigating the objects but it does not allow for controlling the amount of time the rat spends in the arena. The rat is then removed for a retention delay, after which it is returned to the arena, where there are now two new objects –one is identical to the sample and the other is novel. During the test, the amount of time the rat spends investigating the novel object is compared to the amount of time spent investigating the sample object, and an exploratory bias for the novel object on the test is taken to indicate the rat recognizes the sample object. Since it requires several minutes to conduct a single trial, normally only one trial is administered per day. Similarly, when collecting data using a particular delay, typically only a few trials are administered and in some cases only one trial (Gulinello et al., 2018). Consequently, a large number of rats are needed in order to obtain results with acceptable levels of variance.

When Ennaceur and Delacour (1988) first described the series of experiments using this test, they simply referred to it as a "one-trial memory test." Over time, it has been given several names, the most common being the *spontaneous object recognition* (SOR) task, the *novel object recognition* (NOR) test, and the *novel-object-preference* (NOP) test. In the present thesis, I will refer to the task as the novel-object-preference test because this nomenclature most accurately

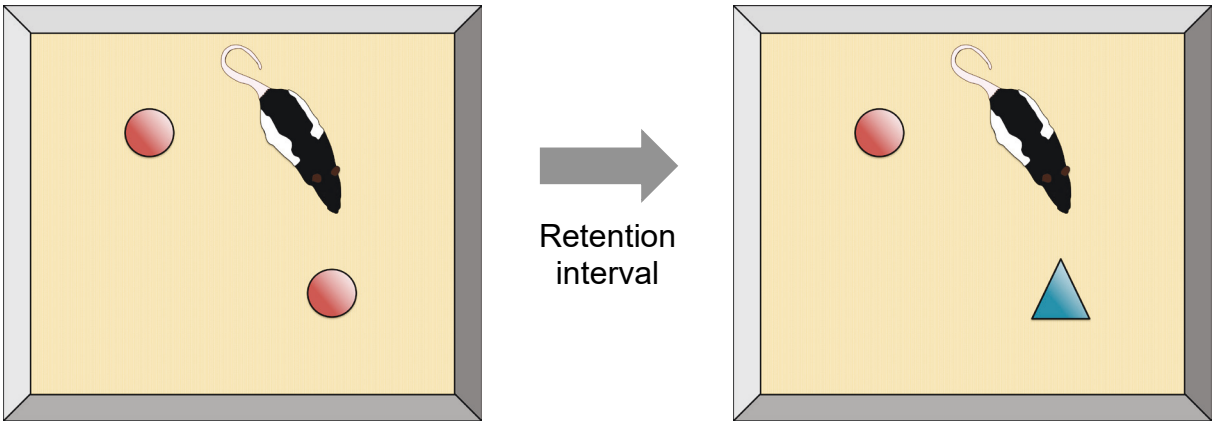

Figure 1.6. Illustration of the NOP test design. The image on the left depicts the familiarization phase (with two copies of the sample object) and the image on the right depicts the test phase (with a third copy of the sample object and a novel object).

reflects the variable being measured, that is, a rat's preference or lack thereof towards a novel object.[4]

Additionally, when reporting NOP findings, I will describe results in terms of whether or not a "novel-object preference" was observed rather than "object-recognition," as the former reflects the directly observable behaviour whereas the latter reflects the experimenter's interpretation of the behaviour.

Ennaceur and Delacour described the NOP test as being advantageous over the extant rodent DNMS tasks primarily for two reasons. First, it removed potential interpretational problems associated with tasks that incorporate a reference-memory component when gauging working-memory (i.e., learning a nonmatching rule and applying it on every trial). Specifically, failure to learn a reward contingency could be ruled out as a potential cause for poor performance on the test. Secondly, it did not require positive reinforcers (food reward), therefore, results could be compared to those obtained on human visual-recognition tasks that do not include an appetitive component. Moreover, removing the appetitive component from the paradigm reduced the potential confound of treatment effects that could disrupt motivational responses to a reinforcer.

Through a series of experiments, Ennaceur and Delacour showed that after giving rats a one-time exposure to a sample object, they exhibited novel-object preferences following delays ranging from 1 min to 4 hr, but that this novelty preference disappeared following a 24 hr delay. This meant that memory for objects that were encountered either several minutes or hours earlier could be measured, introducing the possibility to assess long-term object-recognition memory, something that was unattainable with existing rodent DNMS procedures. Overall, the NOP paradigm made data collection more efficient and feasible compared to the DNMS, as data could be collected from several rats in a matter of days and did not require extensive training from an experienced researcher. This paradigm therefore provided an easy way to measure object-recognition memory in rats, which did not suffer from the same drawbacks as DNMS tasks.

It should be noted that Ennaceur and Delacour did not develop the novelty-preference paradigm but rather expanded on a behavioural paradigm first described by Berlyne (1950). This

[4] The term SOR does not seem suitable because while "object-recognition" can be inferred when a rat displays a novel-object preference on the test, the lack thereof does not necessarily signify a lack of "object-recognition" memory. Similarly, the name NOR suggests the rat recognizes the *novel* object on the test, when in fact, it is the *sample* object that would be recognized.

paradigm was originally used to measure exploratory behaviour in rats as a function of their *curiosity* and *preference* for novelty which was measured by introducing new objects to a familiar environment (Berlyne, 1950). Ennaceur and Delacour modified this procedure by implementing varying delays between a familiarization and test phase in order to introduce a new variable to measure—*retention* of an object. Moreover, the NOP test developed for rats was analogous to the human and nonhuman primate *visual paired-comparison* (VPC) task, which was first used to study visual perception (Fantz, 1956, 1958), and later used as a test of visual recognition in human infants (Fagan, 1970; Fantz, 1964) and nonhuman primates (Gunderson & Sackett, 1984). Briefly, on the familiarization phase of the VPC task, the subject is presented with a stimulus object to look at for a designated amount of time, and then following a delay it receives a test, whereby the familiar stimulus is now paired with a novel one. The dependent measure in this test is the amount of time the subject spends attending to or orienting towards each stimulus. When a subject exhibits a significant bias to look at the novel stimulus more than the familiar one, sample stimulus recognition is inferred. A difference between the VPC and NOP test is that while the VPC task can be described as a measure of *visual* recognition, the NOP test using three-dimensional objects cannot be, as rats can also rely on information from other sensory modalities (tactile and olfactory) when interacting with the objects. The VPC task is more commonly used to assess recognition abilities in infants and nonhuman primates (compared to human adults) due to their inability to verbally report whether or not they recognize previously encountered stimuli. Despite the fact that the VPC task has been developed for use with nonhuman primates, few studies have used it to examine the effects of MTL damage on visual-recognition in nonhuman primates. Those that have, typically include additional measures of object-recognition memory using the DNMS task (Bachevalier, Brickson, & Hagger, 1993; Buffalo et al., 1999; Nemanic, Alvarado, & Bachevalier, 2004; Pascalis & Bachevalier, 1999; Zola et al., 2000). Thus, contrary to research using rodents, the majority of experiments that have assessed the neural mechanisms mediating object-recognition memory in nonhuman primates have relied almost exclusively on the DNMS task.

By the early 2000s, the NOP test had gained popularity in the field of neuroscience as a means to elucidate the neural mechanisms underlying object-recognition memory via experimental manipulations (e.g., lesions and pharmacological). Today, a ‘Web of Science’ database search of peer-reviewed articles published in the last 10 years using the terms

"spontaneous object recognition" or "novel object recognition" or "novel object preference" along with "rat" returns 1,966 peer-reviewed articles (Web of Science, May, 2019). Although the NOP test is currently the most widely used object-recognition test for rodents, there is no standard method for analyzing and reporting results. Thus, before describing the findings from experiments that have used the NOP test to examine the effects of discrete MTL lesions on rats' object-recognition memory, it is necessary to first understand the different methods for analyzing NOP data.

1.3.2.1 Analyzing and reporting NOP test results

Unlike the DNMS task, there is no general consensus on how to measure behaviour on the NOP test. While the dependent measure of the test is consistently the amount of time (in seconds) the rat spends investigating the objects on the test, the operational definition for object "investigation" varies across research laboratories. Based on a review of articles published by authors that have cited Ennaceur and Delacour's 1988 study on at least ten occasions, only the following two behavioural measures are explicitly included in the method sections: 1) the minimum distance the subject's nose should be from the object, which varies between 1-4 cm across articles, and 2) climbing over or sitting on an object is not considered investigation (Web of Science, May, 2019). The majority of publications do not list 'chewing the object' or 'rearing with at least one forepaw touching the object' as a behavioural measure to include or exclude towards object investigation despite the fact that a rat will typically engage in both behaviours during the test. A highly cited protocol paper by Bevins and Besheer (2006) published in the journal *Nature Protocols* does however state "…any contact with mouth" constitutes object investigation (p.1309).[5] Regardless, the lack of a stringent operational definition across research laboratories undoubtedly raises concerns over differences in the recorded amount of time a rat spends investigating objects and consequently the conclusions that will be drawn regarding the status of object-recognition memory. Additionally, considering only a few trials are administered, it limits both the sensitivity and stability of the measure.

Similarly, there are several different methods used across research laboratories to report descriptive statistics for NOP data. Object investigation during each cumulative minute of the

[5] Whether or not "chewing" is indeed part of the operational definition and is simply not explicitly written in the method section in papers is concerning because the texture of an object can produce differences in levels of investigation for two objects when one is made of a chewable substance (e.g., plastic) and the other is not (e.g., glass).

test phase is recorded and typically data from only the first few minutes is analyzed. The rationale for the latter is that investigation during the beginning of the test is presumed to provide the most reliable measure of novelty preference, because as the test phase continues, the novel object becomes more familiar, reducing the differential exploration of the objects (Dix & Aggleton, 1999). Object investigation on the test is reported using one of the following three methods: 1) preference or investigation ratio ($Time_{Novel}/(Time_{Novel} + Time_{Sample})$, 2) discrimination index or difference score ($Time_{Novel} - Time_{Sample}$), or 3) discrimination ratio ($Time_{Novel} - Time_{Sample})/ (Time_{Novel} + Time_{Sample})$. When using the latter two methods, a value of zero indicates equal time spent investigating the sample and novel object, whereas using the first method a ratio of 0.50 indicates equal time spent investigating both objects. The rationale behind using a ratio is that it adjusts for potential individual differences in the total amount of investigation time on the test.

In terms of inferential statistics, as in the case of experimental manipulations when two or more groups of rats are tested, group mean scores can be analyzed using either a "within-subjects" or a "between-subjects" method. The former entails comparing a group's mean score to what would be expected by chance (e.g., a preference ratio of 0.5 or discrimination index of 0), where a group score that is statistically significantly above chance indicates that, on average, the group spent more time investigating the novel object versus the sample object. When this occurs, it is taken to indicate that the rats recognized the sample object, and thus object-recognition memory is intact. This method treats NOP scores as binary data (yes/no investigation bias). In contrast, the "between-subjects" method involves comparing the group mean scores to one another (e.g. treatment group vs. control group). When a statistically significant difference is observed between group scores, the group with the lower mean score is considered to have an object-recognition impairment—even if the group score is significantly above chance. Accordingly, using this method, the implication is that the magnitude of the novel-object preference is directly proportional to the strength of the memory for the sample object.

The majority of research publications report both the between-subjects and within-subjects analysis, whereas the interpretation of the results and conclusions regarding the effects of an experimental treatment on object-recognition are typically based solely on the between-subjects analysis. Given that there are two methods used to interpret behaviour on the NOP test, it is unsurprising that different conclusions can be drawn from the same set of data. In order to

comprehend the interpretational problems associated with the way NOP data are analyzed, it will help the reader to be familiar with research findings using the NOP test. The following section presents results from experiments that have used the NOP test to examine the effects of lesions made to MTL structures on rats' object-recognition memory. Given that there are two methods used to interpret NOP test scores, I will highlight which statistical method was used when presenting the results in order to provide context for the authors' conclusions.

1.3.2.2 Examining the effects of MTL lesions on rats' NOP

The majority of research aimed at testing the effects of HPC damage on object-recogniton memory has relied almost exclusively on using the DNMS task, not the NOP test. Of the studies that have been conducted using the NOP test, they support the DNMS findings that an intact HPC is not critical for normal object-recogntion memory. Similar to control rats, rats with HPC lesions still exhibit significant novel-object-preferences on tests following delays lasting several minutes to 24 hr (Cohen & Stackman, 2015; Ennaceur & Aggleton, 1994, 1997; Ennaceur, Neave, & Aggleton, 1996, 1997; Forwood, Winters, & Bussey, 2005; Gaskin, Tremblay, & Mumby, 2003; Mumby, Gaskin, Glenn, Schramek, & Lehmann, 2002; Winters, Forwood, Cowell, Saksida, & Bussey, 2004). One group of researchers, however, reported that rats with HPC lesions displayed NOP deficits relative to control rats following delays lasting longer than 10-min, which the authors interpreted as resulting from object-recognition deficits (Clark, Zola, & Squire, 2000). In this case, the HPC group had average scores that were significantly lower relative to the control group, but their scores were significantly above chance, indicating that they successfully discriminated between the novel and sample object on the test (even after a 24-hr delay). Despite this report, most researchers that have conducted experiments aimed at testing the hypothesis that HPC damage impairs object-recognition memory have failed to find support for it. Overall, interpreting the effects of HPC damage on object-recognition memory using the NOP test has been fairly straightforward because rats with HPC lesions perform like control rats.

Unlike research examining the role of the HPC in object-recognition memory, the majority of behavioural experiments assessing the effects of PRh damage on rats' object-recognition memory have relied almost exclusively on the NOP test, or variants of it. The findings from these studies have largely concluded that the PRh plays a critical role in object-recognition memory (for a review see Brown, Barker, Aggleton, & Warburton, 2012; Murray &

Richmond, 2001; Winters et al., 2008). The conclusions from these studies are exclusively based on between-subjects analyses, which have shown that rats with PRh lesions have significantly lower novelty preference scores compared to control rats following delays lasting several minutes up to 48 hr (Aggleton, Keen, Warburton, & Bussey, 1997; Barker & Warburton, 2011; Bussey, Duck, Muir, & Aggleton, 2000; Bussey, Muir, & Aggleton, 1999; Ennaceur & Aggleton, 1997; Ennaceur et al., 1996; Liu & Bilkey, 2001; Murray & Richmond, 2001; Norman & Eacott, 2004; Winters & Bussey, 2005; Winters et al., 2004).

Although these studies reveal that rats with PRh lesions exhibit lower average preference scores relative to control rats, the results are inconsistent in terms of whether or not these scores are significantly above chance. Upon closer inspection of the within-subjects analyses, some reveal that rats with PRh lesions fail to exhibit significant novel-object preferences on the test following delays lasting up to 15 minutes (Aggleton et al., 1997; Barker & Warburton, 2011; Ennaceur & Aggleton, 1997; Ennaceur et al., 1996; Liu & Bilkey, 2001), whereas others show intact novelty preferences, despite being significantly lower than those of control rats (Bussey, Duck, Muir, & Aggleton, 2000; Bussey et al., 1999; Ennaceur et al., 1996; Ennaceur & Aggleton, 1997; Norman & Eacott, 2004, 2005). In fact, rats with PRh lesions have been shown to exhibit significant novel-object preferences following delays lasting one hour (Barker, Bird, Alexander, & Warburton, 2007; Norman & Eacott, 2004) and even up to 24 hours (Winters et al., 2004). These findings demonstrate that rats with PRh damage can successfully discriminate between novel and sample objects on the test, indicating that they recognize previously encountered objects.

The discordant findings make it difficult to firmly conclude whether or not PRh lesions disrupt rats' object-recognition abilities. What can be concluded with more certainty from these findings is that PRh lesions appear to affect a rat's spontaneous tendency to explore novel objects. The extent to which the latter reflects an object-recognition deficit, however, remains unclear. Accordingly, this raises two inherent issues surrounding the use of the NOP test as a gauge for object-recognition abilities: 1) to what extent does the magnitude of a novel-object preference reflect the persistence or accuracy of a rat's memory for the sample object? and 2) what does a lack of novel-object preference signify in relation to the status of object-recognition memory?

Despite the widespread use of the NOP test as a gauge of object-recognition abilities, efforts to answer these two questions have been minimal. The following section details the research that has attempted to answer these questions, and illustrates the problems associated with the way NOP results are typically interpreted.

1.3.2.3 Pitfalls of the NOP paradigm

The underlying assumption when applying the between-subjects statistical method is that higher novelty preferences are indicative of superior memory for the sample object. This is problematic, however, because to date there is no research indicating that the *magnitude* of a rat's novel-object preference reflects the persistence or accuracy of its memory for the sample object. For instance, the amount of time rats spend investigating objects during the familiarization phase (and presumably encoding object features) does not predict the degree of their novel-object preference, nor does providing rats with prolonged or repeated exposure to a sample object affect preference magnitude (Gaskin et al., 2010; Gervais et al., 2013, 2016; Gulinello et al., 2018). In one study, groups of rats were given different amounts of time to investigate a sample object: 5, 30, 60, 90, or 120 s, and were tested 3 hours later (Gaskin et al., 2010). The rats in the first three groups failed to show a significant novel-object preference on the test and their mean scores did not significantly differ from one another. Conversely, rats that spent 90 or 120 s investigating objects during the familiarization phase displayed significant novel-object preferences, but their mean scores were not significantly different from one another. The lack of a linear relationship between the amount of time spent investigating sample objects and subsequent novelty preference scores suggests the latter may not truly represent strength in object-recognition memory. In a second experiment by the same researchers, rats were given repeated exposure to the same sample object over several trials, each time presented alongside a different novel object. They found that novel-object preferences did not significantly increase over trials, suggesting that repeated exposure to the sample object does not increase novelty preferences (Gaskin et al., 2010). These findings are contrary to two major assumptions that underlie the manner in which NOP data are usually interpreted –1) that rats are encoding information about the sample object's features when investigating it during the familiarization phase, and 2) the magnitude of the novel-object preference is a reflection of the persistence or accuracy of a rat's memory for the sample object. One implication of these findings is that

differences in the magnitude of a novelty-preference should not be uncritically taken to reflect differences in memory ability.

Given the abovementioned findings, it is apparent that using the within-subjects analysis to interpret NOP results seems more appropriate. Indeed, when a group shows a significant bias to explore a novel object over a sample object, one can be confident that on average, the group recognized the sample object (presuming other factors have been controlled for such as counterbalancing objects between rats). In this respect, interpreting what a significant novelty preference on the test signifies is rather straightforward. However, a problem arises when trying to interpret what a *lack* of novelty-preference on the test signifies in relation to the status of object-recognition. A common implicit assumption is that the group, overall, has an object-recognition memory impairment. This interpretation, however, is an example of an inverse fallacy: if *X*, then *Y*; if not *X*, then not *Y*. When a rat exhibits a significant novelty preference on the test, it can be confidently taken to indicate intact memory for the sample object, however, the inverse—a failure to exhibit a novelty preference on the test—does not confirm with the same level of certainty the status of memory for the sample object. As with any test of incidental learning in which an animal spontaneously explores, it is challenging to interpret what a behavioural response signifies in relation to some internal construct, such as memory. Indeed, a lack of bias to explore novel objects on the test following some treatment may reflect an object-recognition impairment, but it could also reflect a disruption in non-mnemonic processes. For example, the treatment may alter or suppress a rat's natural exploratory response towards familiar and unfamiliar objects (e.g., increase neophobia), or it could increase a behaviour that is incompatible with object exploration (e.g., thigmotaxis—staying close to the walls of an open field apparatus), thus reducing exploratory discrimination and obscuring intact recognition abilities. For this reason, caution should be exercised when inferring the meaning of null preferences following some form of treatment.

The number of potential factors for why a rat fails to display a novelty preference on the test is confounded by the fact that the NOP test does not involve a goal, and thus the rat is not required to make an explicit choice response based on memory. The lack of an instrumental response is a shortcoming of the NOP test because it makes it difficult to rule out alternative explanations other than an object-recognition memory impairment as the reason a rat fails to exhibit a novel-object preference on the test. On the familiarization phase, the rat is likely

encoding information not only about the sample objects but also information on their location relative to spatial cues and the surrounding context.[6] On the test phase, rats may rely on spatial and nonspatial information to successfully discriminate between the sample and novel object. Thus, when a rat fails to display a novel-object preference following some treatment, it could reflect a disruption in memory for a number of factors related to information learned during the familiarization phase. For example, the treatment may have disrupted memory for the sample object, the particular context in which the object was encountered (e.g., room and apparatus), or even the specific location of the sample object relative to cues within the apparatus (including the second copy of the sample object). This raises concerns about potential confounds that are introduced when trying to estimate a rat's memory for a previously encountered object when it is not required to make an explicit choice between familiar and unfamiliar objects. Indeed, this may explain why there are inconsistent findings across NOP studies following some treatment.

Moreover, a rat's spontaneous investigation of a novel object can be modulated by internal attributes, such as stress levels, whereby higher stress levels reduce the tendency to approach novel objects (Gulinello et al., 2018; Hughes, 1997), or by external attributes related to test conditions (Berlyne, 1955; Besheer & Bevins, 2000; Blaser & Heyser, 2015; Ennaceur, 2010; Wilkinson, Herrman, Palmatier, & Bevins, 2006). For example, object attributes such as size, texture, and complexity can induce different amounts of investigation (Berlyne, 1955; Chemero & Heyser, 2005; Ennaceur, 2010; Heyser & Chemero, 2012). Objects that can be climbed over and ones that have complex features elicit greater levels of exploration that do not decline with successive presentations compared to objects that cannot be climbed on or have simple features (Berlyne, 1955; Chemero & Heyser, 2005; Heyser & Chemero, 2012). Moreover, increasing the amount of exposure to the testing environment prior to the familiarization phase increases sample object investigation during the familiarization phase. The latter is thought to occur because other competing behaviours, such as exploring features of the apparatus, are reduced (Besheer & Bevins, 2000; Sheldon, 1969). These findings reveal the delicate nature of the testing conditions when using the NOP test and highlight the importance of standardizing test

[6] Indeed, there are variants of the NOP paradigm used to measure such aspects of recognition memory. For example, *object-place memory*—the ability to recognize that a familiar object is in a location where there previously was no object (Dix & Aggleton, 1999; Ennaceur et al., 1996; Gaskin, Gamliel, Tardif, Cole, & Mumby, 2009; Mumby, Gaskin, et al., 2002) and *object-context memory*—the ability to recognize that an object was previously encountered in a particular context (Dellu et al., 1997; Dix & Aggleton, 1999; Mumby, Gaskin, et al., 2002; O'Brien et al., 2006; Piterkin et al., 2008).

conditions across studies. Additionally, the number of potential factors that can influence behaviour on the test demonstrate how it can be difficult to conclude with certainty that a lack of novel-object preference reflects a failure in object-recognition memory, and not some alternative explanation.

In summary, it is unclear what the magnitude of the novelty preference signifies in relation to the status of object-recognition memory, despite the widespread use of this measure as an indicator for object-recognition abilities. Moreover, because the NOP test does not require the rat to make an explicit choice response based on memory, it is difficult to rule out alternative interpretations for the observed behaviour on the test. Accordingly, the interpretational problems associated with the NOP test call into question the internal validity of it as a means to measure object-recognition memory in rats.

1.4 Rationale and objectives of the thesis

Although the existing DNMS tasks can provide a fairly precise estimate of a rat's object-recognition abilities, there are certain drawbacks associated with them. Specifically, rats require extensive training to learn the nonmatching rule, they are difficult for inexperienced experimenters to effectively employ, and rats' scores on the test decline when using delays lasting more than a few minutes. Consequently, conventional DNMS tasks are labour intensive and cannot be used to assess long-term object-recognition memory. The NOP test on the other hand is easy to use, yet it suffers from inherent interpretational problems, which raise concerns regarding its internal validity. Given the limitations associated with both tasks, the aim of this thesis was to develop a new object-recognition task for rats. There were four objectives developed for this task to address the known limitations of the existing tasks, namely, we wanted to create a task that: 1) rats could master quicker than conventional DNMS tasks, 2) was simple for the experimenter to employ, 3) could be used to assess long-term object-recognition memory, and 4) provided a straightforward interpretation of behaviour as it relates to object-recognition memory. The interpretational problems associated with the NOP test would likely apply to any test of incidental learning that lacks an unambiguous instrumental behavior. Accordingly, we decided to develop a modified DNMS (mDNMS) task that would incorporate the advantageous features of conventional DNMS tasks, yet would be easier for rats to acquire and less difficult for experimenters to use. A secondary goal of the thesis was to compare rats' performance on the new task to that on the NOP test as a means to validate the latter.

In the experiment described in Chapter 2, rats were trained on the mDNMS task, and then their performance on the task was assessed following various retention intervals. Rats required significantly fewer trials to learn the nonmatching rule compared to conventional DNMS tasks, and their scores showed good test re-test reliability. The same rats' exhibited significant novelty-preference scores on the NOP test, however their scores showed poor test re-test reliability and were not significantly correlated with mDNMS scores. The latter finding suggests that the two tasks may not necessarily tax similar underlying cognitive processes.

The experiments described in Chapter 3 assessed rats' performance on the mDNMS task following long retention intervals (72 hr, 3 weeks, and ~45 weeks), while at the same time measuring their performance on the NOP test using the same delays. Rats successfully discriminated between the novel and sample objects on the mDNMS task following all three delays, whereas on the NOP test, rats failed to exhibit significant novel-object preferences. The divergent findings between rats' performance on both tasks further add to concerns regarding the way NOP data are typically interpreted, such that the magnitude of the novel-object preference reflects the persistence or accuracy of the memory for the sample object.

The experiment described in Chapter 4 examined the effects of separate lesions made to the HPC and PRh on mDNMS task performance and the NOP test following short retention intervals. Neither HPC nor PRh lesions failed to disrupt performance on the mDNMS task, but rats with PRh lesions failed to display a novelty preference on the NOP test. The discrepancy in the PRh rats' performance on both tasks raises concerns regarding the internal validity of the NOP test, specifically, that a lack of novelty preference is not necessarily indicative of an object-recognition memory impairment.

The aim of the experiment in Chapter 5 was to refine the mDNMS task. We incorporated a Go/No-go procedure to include *latency to make a choice* as a behavioural measure of object-recognition memory. The findings revealed that latency to make a choice provided a more sensitive measure of object-recognition memory than choice-accuracy on the test. The findings from this experiment demonstrate the advantages of incorporating multiple behavioural measures as a means to estimate object-recognition memory in rats.

Chapter 2: A new test of object-recognition memory for rats

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Abstract

Studies of object-recognition memory in lab rats began in the late 1980s, using variants of the trial-unique delayed nonmatching-to-sample (DNMS) task. By the end of the 20th century, most investigators who wanted to study object-recognition in rodents had abandoned the DNMS task in favor of the novel-object-preference (NOP) test, mainly because the latter test is relatively easy to employ, whereas conventional DNMS tasks are not. Some concerns have been raised, however, about the internal validity of the NOP test as a method of measuring object-recognition abilities. Specifically, preference scores on the NOP test may not accurately reflect the underlying construct—object-recognition memory. The primary goal of this experiment was to develop a new object-recognition task that addresses the drawbacks of the existent tasks. We developed a modified DNMS (mDNMS) task which included the same underlying principles of DNMS tasks, namely the inclusion of an instrumental response and a reward for accurate choices, while using a large apparatus that reduced both the involvement of the experimenter in administering individual trials and constraints on natural exploratory behaviour. Rats were trained on the mDNMS task and performance was examined using several short delays ranging from 100 s to 10 min. Rats successfully learned the nonmatching rule in fewer than 25 trials, and they made accurate choices with retention intervals of up to 10 min. A secondary goal of the experiment was to compare rats' performance on the mDNMS task to scores obtained on the NOP test as a means to validate the latter. Presuming scores on the NOP test are an index of a rat's object recognition abilities, we predicted to find a positive linear correlation between scores on the mDNMS task and NOP test. The results revealed that there was no correlation between scores on the mDNMS task and the NOP test, indicating that scores on the two tasks do no necessarily reflect the same underlying construct. Overall, the results from the new task provide support for its effectiveness as an estimate of object-recognition memory in rats. Moreover, the findings raise concerns regarding the use of the NOP test as a means to measure object-recognition abilities.

2.1. Introduction

Two behavioral paradigms have been used to assess object recognition—the ability to discriminate the familiarity of previously encountered objects—in rats: *delayed nonmatching-to-sample* (DNMS), using trial-unique or pseudo-trial-unique stimuli, and *novel-object preference* (NOP). Variants of the NOP test are the most widely used, by far. Concerns have been raised, however, about the internal validity of the NOP test as a method of measuring object-recognition abilities (Gaskin et al., 2010; Gervais et al., 2013, 2016); these concerns are outlined, below. Although DNMS tasks have not been subjected to the same criticisms, they possess different shortcomings that limit their usefulness; namely, compared to the NOP test, conventional DNMS tasks are considerably more difficult and time-consuming to employ.

The limitations inherent in the NOP test and conventional DNMS tasks motivated us to develop a new method for assessing object-recognition memory in rats. In order to appreciate the merits of the new procedure, it is important to first examine the existing methodologies for assessing object-recognition memory in rats, and examine their respective advantages and limitations.

Various DNMS procedures were developed in the late 1980s (e.g. Aggleton, 1985; Mumby, Pinel, & Wood, 1990; Rothblat & Hayes, 1987). Each variant uses a somewhat different apparatus, but all use 3-dimensional objects for test stimuli, and follow the same general procedure: On each DNMS trial, a sample object is briefly presented (usually for only a few seconds or less), and after a retention interval, the sample is presented again along with a novel object (i.e., one the rat has not previously encountered during the current session). The rat receives a reward if it selects the novel object. Different sample and novel objects are used on each trial, so reliably accurate performance requires that rats can recognize the sample objects. Memory demands are manipulated by varying the retention interval or the number of objects to remember on each trial. There are several trials per session, and a well-trained rat may be able to complete 20 or 25 trials in less than half an hour (if the retention interval on each trial is only a few seconds, and the inter-trial interval is similarly brief). If rats that receive different treatments consistently perform at similar levels of accuracy when memory demands are minimal, then it can be inferred with some confidence that different levels of accuracy under more challenging conditions reflect real differences in object-recognition abilities.

Although a DNMS task can provide a fairly precise estimate of a rat's object-recognition

abilities, the DNMS procedures developed in the 1980s share some drawbacks in common: They are difficult for inexperienced investigators to employ effectively, primarily because the experimenter is in the room with the rat and plays an active and ongoing role in administering the trials (Herremans et al., 1995; Mumby, 1995; Mumby, Kornecook, et al., 1995). Even in the hands of a capable experimenter, most rats require hundreds of trials before they reach peak performance, which can require weeks of daily training. Many investigators would consider this time-requirement to be prohibitively long. Moreover, even after extensive training, rats can perform accurately only if the retention delay is no more than a few minutes, and for this reason, most previous studies have used maximum retention delays of 120 - 300 s (see Table 1 in Appendix A). Thus, conventional DNMS tasks cannot be used to study long-term memory with retention intervals of several minutes, or hours, or days.

Conventional NOP procedures vary slightly from one laboratory to another, but all are generally similar to those described by Ennaceur and Delacour (1988). A rat is placed in an arena, where it is allowed to explore and investigate two identical objects for a few minutes. The rat is then removed for a retention delay, after which it is returned to the arena, where there are now two new objects -- one is identical to the sample and the other is novel. Rats tend to spend more time investigating the novel object during the test, indicating that they recognize the sample object. With conventional procedures, rats may show a novel-object preference after retention intervals of up to 24 hr, and with modified procedures, after intervals of up to several weeks (Gaskin et al., 2003; Mumby, Glenn, Nesbitt, & Kyriazis, 2002; Mumby, Piterkin, Lecluse, & Lehmann, 2007; Mumby, Tremblay, Lecluse, & Lehmann, 2005). Thus, the NOP test has the potential to assess long-term object-recognition memory after retention intervals of several days.

The NOP test exploits rats' tendency to investigate novel objects more than familiar objects, when those objects are encountered in a familiar environment (Berlyne, 1950; Besheer & Bevins, 2000). Because the novelty preference is displayed spontaneously under appropriate conditions, no extensive training is required for either experimenters or rats, which makes the NOP test a widely accessible and time-efficient procedure for generating data with the potential to provide insight into rats' object-recognition abilities.

Despite the practical advantages of the NOP test, however, some recent observations have raised concerns about the internal validity of the NOP test as a gauge for object-recognition

abilities. For instance, the amount of time rats spend investigating objects during the familiarization phase does not predict the magnitude of their novel-object preference, nor does providing rats with prolonged or repeated exposure to a sample object affect preference magnitude (Gaskin et al., 2010; Gervais et al., 2013, 2016). These findings are contrary to two major assumptions that underlie the manner in which NOP data are usually interpreted -- 1) that rats are encoding information about the sample object's features when investigating it during the familiarization phase, and 2) the magnitude of the novel-object preference is a reflection of the persistence or accuracy of a rat's memory for the sample object. One implication is that differences in the magnitude of a novelty-preference should not be uncritically taken to reflect differences in memory ability.

The interpretational problems associated with the NOP test would likely apply to any test of incidental learning that lacks an unambiguous instrumental behavior. Accordingly, we decided early in our plan to develop a new object-recognition test that should incorporate some advantageous features of conventional DNMS tasks: 1) the involvement of an instrumental response with which the rat makes an explicit choice between familiar and unfamiliar objects, 2) a reward for accurate choices, and 3) the possibility of testing individual rats on several trials per session, with each trial consisting of an independent test of recognition memory. A new object-recognition task would be appealing only if it were easier for rats to learn than the DNMS tasks developed in the 1980s. We reasoned that the latter objective could be achieved by diminishing the presence and role of the experimenter in administering individual trials. What we came up with is essentially a modified-DNMS (mDNMS) task. There were three objectives when we developed this task, namely, we wanted a DNMS task that rats could master quicker than conventional DNMS tasks, required little human intervention when administering trials, and could successfully be used to assess long-term object-recognition memory in rats.

Rats were trained on the mDNMS task and then tested using retention delays ranging from 100 s to 4 hr. We also tested rats on the NOP test using a 180-s delay. A second major goal of this study was to compare rats' performance on the mDNMS task to scores obtained on the NOP test, as a means to validate the latter. Indeed, if we accept that scores on the mDNMS task accurately reflect rats' recognition abilities, and this is what supports good performance on the task, and the NOP test is a valid measure of object-recognition abilities, then we predicted a positive linear correlation to exist between scores on the mDNMS task and NOP test.

2.2. Materials and Method

2.2.1. Subjects

The subjects were ten experimentally naïve male Long-Evans rats (Charles River, St. Constant, QC), weighing 225-275 g (~8 weeks old) at the start of the experiment. The rats were pair-housed in polypropylene cages (48 × 25 × 20 cm) in a colony room maintained under a reverse 12:12 light-dark cycle, with light onset at 8:00 p.m. The rats had continuous access to water and each received a daily ration of ~25 g of rat chow (Charles River Rodent Animal Diet, no. 5075) in the late afternoon, after behavioral testing was finished for the day. All procedures were approved by the Concordia University Animal Care and Use Committee, and were in accordance with the guidelines of the Canadian Council on Animal Care.

2.2.2. Apparatuses

2.2.2.1. mDNMS task. A large multi-level environment (152 × 145 × 86 cm) was used to test the rats (Figure 2.1). The apparatus was a modified, freestanding steel cage rack, enclosed on three sides by wire mesh, with a removable, clear acrylic front panel. The apparatus had five levels, each covered with woodchip. The top four levels were divided into two equal halves by a plastic barrier wall, and the bottom level remained undivided. A loading cage (58 × 37 × 20 cm) was placed on the top left side of the apparatus. A rat entered the apparatus via a hole in the bottom of the loading cage that was placed over a passageway leading to the top level of the apparatus. Rats traversed the different levels via wire mesh passageways located on both sides of the apparatus. The design of the apparatus was such that a rat had to climb down the passageways on the left side of the apparatus in order to gain access to the right side, which it then could ascend from level to level. The top four divided levels contained plastic rectangular platforms (30 × 12 × 1 cm) each with a recessed food well (2 cm in depth), over which stimulus objects could be placed. One platform was placed on each level on the left side of the apparatus, and on the right side of the apparatus, two platforms were placed on each level with the food wells 9 cm apart. All platforms were positioned near the middle barrier wall, in line with the passageway that provided access to the level. The room contained dim lights (20 lx) and three video cameras were used to record test sessions –one was positioned in front of the apparatus and two were positioned on the test side.

2.2.2.2. NOP test. The apparatus for the NOP test was an open-field arena (60 × 70 × 70 cm), constructed of grey PVC plastic. The floor of the arena consisted of a stainless-steel tray

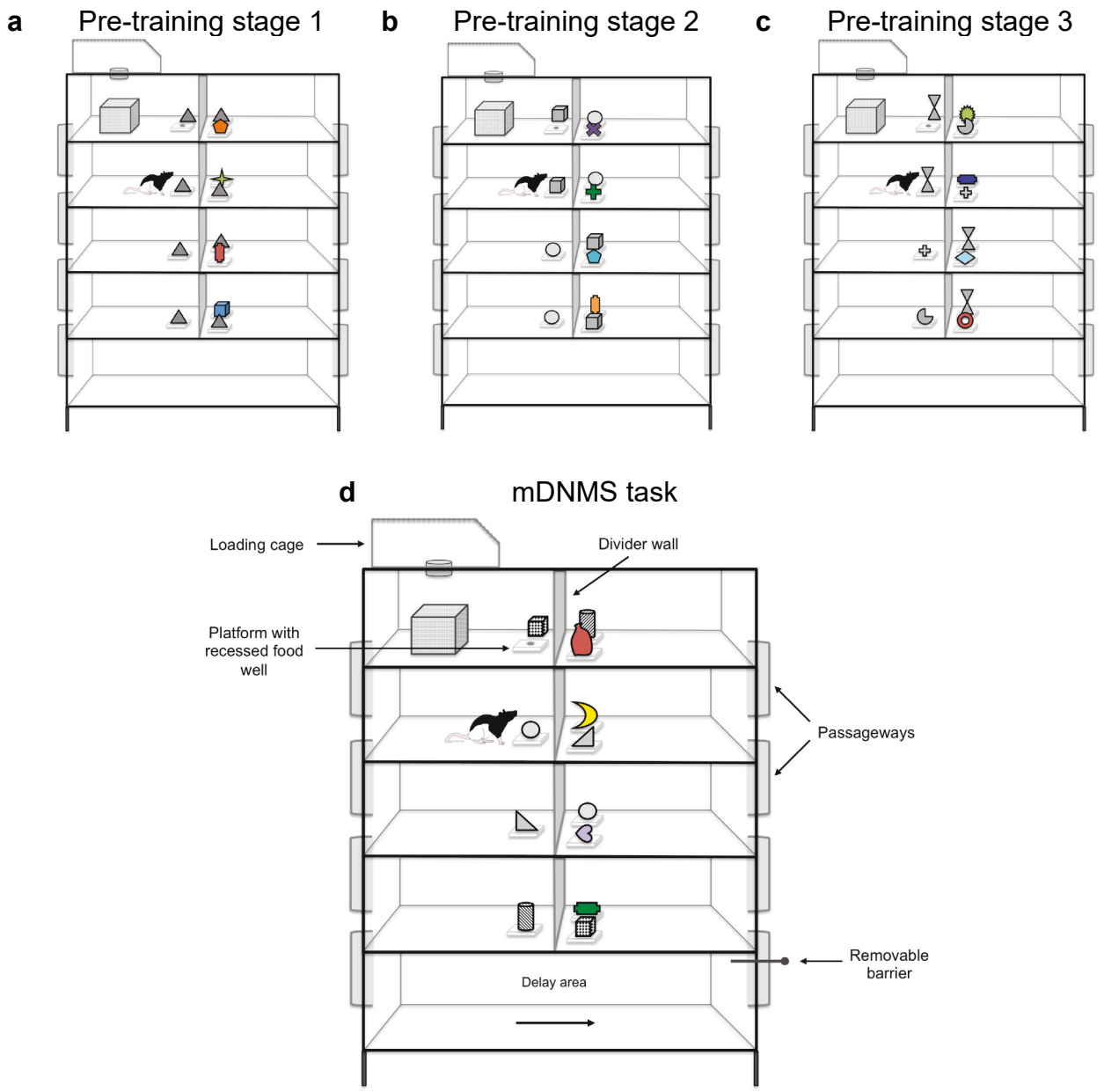

Figure 2.1. Diagram of the apparatus used for mDNMS testing depicting a session on (a-c) Pre-training stage 1, 2, and 3, respectively, and (d) mDNMS task acquisition, training at progressively longer delays, and pseudo-mixed delay testing. A loading cage provided access to the apparatus, and passageways on both sides of the apparatus allowed rats to access the different levels. The top four levels contained plastic platforms with recessed food wells in which objects could be placed over. During pre-training, the rat descended the left side of the apparatus encountering either (a) four copies of one sample object on stage 1, (b) two copies of two distinct sample objects on stage 2, or (c) two copies of one sample object and two distinct sample objects on stage 3. During (d) mDNMS task acquisition and subsequent testing, the rat encountered four unique sample objects as it descended the left side. For all stages (a-d) once the rat reached the bottom level, it traversed to the right side where it ascended each level encountering 4 different tests. On each test a copy of the sample object was paired with a unique novel object. During training at progressively longer delays and pseudo-mixed delay testing, a delay was imposed by temporarily blocking access to the test by inserting a removable barrier into the passageway.

covered with woodchips. The floor was removable via a slot at the bottom of one wall to facilitate changing the woodchips between each trial. The testing room contained dim lights (14 lx) and a videocamera was positioned over the arena to record the sessions for later analysis.

2.2.2.3. Stimulus objects. A total of 285 different objects were used as stimuli for the mDNMS task. Objects were made of plastic, metal, glass, or glazed ceramic, and ranged in size from 4 to 18 cm in height, and 4 to 13 cm in width. Each object was large enough to cover the food well but light enough to be easily displaced by a rat. There were two copies of each mDNMS task object –one for the learning phase and one for the test. The objects were cleaned after every trial on which they were used, by wiping with a damp paper towel. At the end of each day the mDNMS task objects were cleaned using a diluted bleach solution (1:20 concentration ratio).

A separate pool of 6 objects was used for the NOP test. Objects for the NOP test were made of plastic, metal, glass, or glazed ceramic, and ranged in size from 7 to 18 cm in height, and 5 to 13 cm in width. There were at least three copies of each object–two for the familiarization phase and one for the test. A small glass jar (6 cm high) was attached to the bottom of each object with epoxy. The objects were fixed in place by screwing the jars into inverted lids that were attached to the stainless-steel tray in the open field arena (positioned 27 cm from opposing corners). The objects were cleaned after every trial on which they were used, by wiping with a damp paper towel. At the end of each day the NOP objects were cleaned using a 70% ethanol solution.

2.2.3. Behavioral procedures

2.2.3.1. mDNMS task. The new paradigm involves a series of training stages, each of which the rat had to reach a specific criterion before moving onto the next stage. There were three stages: 1) habituation, 2) pre-training, and 3) mDNMS task acquisition.

2.2.3.1.1. Habituation. The rats were handled for ~10 minutes daily for one week before they were habituated to the apparatus. The goal of habituation was to have rats complete an entire circuit of the apparatus (start on the top left level and finish on the top right level), with relatively little hesitation. Rats received two habituation sessions per day. On the first 20 sessions, all 10 rats were placed in the apparatus for 30 minutes with no stimulus objects present, and ~20 Cheerios (1.8 g, General Mills) were placed on each level near and inside the food wells. On the final two habituation sessions, the rats were placed in the apparatus in pairs for 5

minutes, and only 5 Cheerios were placed inside each food well. By this point, each rat was consistently eating Cheerios from each food well and reliably completing the entire circuit. During this stage and subsequent stages, the experimenter left the room after placing the rats in the apparatus and watched the session on a TV monitor in an adjacent room.

2.2.3.1.2. Pre-training. Pre-training consisted of three stages; stage 1, 2, and 3 (Figure 2.1a-c). The rats were now tested individually and they were introduced to stimulus objects. The purpose of the pre-training stages was to gradually familiarize the rats to the procedural aspects of the task (e.g., learn to displace objects from over food wells and to dig for a buried Cheerio placed in food wells) and to teach them that the visual/tactile object features were key to predicting food location. Pre-training stage 1, 2, and 3 differed in the number of distinct sample objects that were presented to the rat: one, two or three, respectively. The rat had to reach a performance criterion at each stage before advancing to the next stage.

On each stage, a rat received one session per day, which consisted of two phases: a sample phase and a test phase. On the sample phase, the rat descended the left side of the apparatus and encountered either four copies of one sample object (stage 1, see Figure 2.1a), two copies of two different sample objects (stage 2, see Figure 2.1b), or three different sample objects –two copies of one object on the top two levels, and two distinct sample objects on the bottom two levels (stage 3, see Figure 2.1c). One Cheerio was placed in each food well to encourage the rat to approach and investigate the sample objects. On the test phase, the rat ascended each level on the right side of the apparatus encountering a different novel object paired with a copy of the sample object and one Cheerio was placed in the food well under each novel object. Thus, the test phase consisted of four separate 'trials', one for each level. On stage 2 and 3 the sample objects on the test phase were presented in the same order that the rat had encountered them on the sample phase (i.e., the first sample object appeared on the first test level). The sample and test phase were separated by a short retention interval in which the rat spent traversing the bottom level of the apparatus. On the first few stage 1 sessions, the objects only partially covered the food well to encourage timid rats to displace objects. As sessions continued, the objects were gradually positioned to cover the entire food well. During stage 2, the Cheerios on the sample and test phase were gradually buried beneath woodchip until the food well was entirely filled to the top (2 cm deep) and rats were consistently digging for the Cheerio. Burying the Cheerio was done in an attempt to reduce the likelihood that a rat would rely on

olfactory cues to locate the reward. Moreover, by making the rat dig for the Cheerio we increased both the delay and amount of effort necessary to retrieve the Cheerio from beneath the novel object.

A correct choice on a test trial was defined as the rat either displacing the novel object before displacing the sample object, or only displacing the novel object. An incorrect choice was defined as the rat only displacing the sample object, or displacing the sample object before the novel object. If a rat did not displace either object on a particular test, it was considered a non-trial. For a particular rat, we began recording its accuracy on the test phase once all objects fully covered the food well. Different sample and novel objects were used on each session. On stage 1 a total of 15 different object sets were used, each containing 8 copies of one sample object and 4 unique novel objects. After 15 sessions, rats re-encountered the objects again in the same sequence, starting with the first object set. On stage 2 and 3 four new object sets were introduced –each containing four copies of two distinct sample objects and four unique novel objects. These objects sets were used in combination with the stage 1 sets. The location of the novel object on the test phase was counterbalanced in a pseudorandom order.

After the rat completed the final test, the experimenter entered the room and removed the rat from the top right side of the apparatus. Between each rat, the woodchip on every level was redistributed to spread any potential odor cues left by a previous rat and each object platform was cleaned using a 70% ethanol solution. A rat advanced to the next pre-training stage once it reached a performance criterion of at least 80% of trials correct on five consecutive sessions (i.e., at least 16 correct trials out of 20 trials). A rat was given a maximum of 50 sessions at each stage to reach the performance criterion.

2.2.3.1.3. mDNMS task acquisition. During the final training stage, rats encountered four distinct sample objects, one on each of the divided levels of the sample phase (see Figure 2.1d). Thus, this stage was similar to conventional DNMS tasks in that each sample object was encountered only once during the sample phase and was subsequently paired with a unique novel object for the test phase. Similar to pre-training, a session consisted of a sample and test phase. On the sample phase, a rat descended the apparatus to familiarize itself with four distinct sample objects, encountering a different one on each level. One Cheerio was buried in the food well under each sample object. During the test phase, a copy of each sample object was presented

next to a novel object. A Cheerio was buried under the novel object on each test level. Each session consisted of four trials, as there were four distinct sample objects in the apparatus.

From this point forward a new collection of object sets was used. The objects changed on each session, however, the same objects served as the sample objects and novel objects for all rats. Once a particular object was used on a session, it was not used again until all of the objects in each set were used. This resulted in a particular object re-occurring approximately every 20 sessions. Moreover, an object that served as a sample the first time a rat encountered it, served as a novel the next time it was encountered (and vice versa). The sample and novel object on each trial were paired based on similarities in size, weight, and material. The location of the novel object on each test was counterbalanced in a pseudorandom order. A rat was required to reach a performance criterion of at least 80% of trials correct on five consecutive sessions (16 trials correct out of 20). The average delay between the sample and test phase was 30 s (s = 22.47). A rat was given a maximum of 50 sessions at this stage to reach the performance criterion. Rats received one session per day and were tested no fewer than five days per week. The dependent measures were mean percent correct choices and mean number of sessions required to reach the performance criterion.

2.2.3.1.4. Training at progressively longer delays. Once a rat met the performance criterion at the 30-s delay, the delay between the sample and test phase was increased to 70 then 90, 220, 330, 440, and then 630 s.[7] To impose a longer retention delay, the passageway leading to the first test was blocked with a removable barrier (see Figure 2.1). Additionally, after a rat reached the bottom level (delay area), the passageway leading to the last sample object was blocked to prevent a rat from going back to the sample phase. At the end of a particular delay the experimenter entered the room and unblocked the passageway leading to the first test to allow the rat to start the test phase. For each delay, the rat was required to reach the same performance criterion as before (16 trials correct out of 20 trials), or it received a maximum of 30 sessions.

2.2.3.1.5. Pseudo mixed-delay testing. The final stage of DNMS testing consisted of presenting sessions with different retention delays (100, 220, 330, and 630 s) in a mixed fashion. The goal was to compare performance across the range of retention delays used during the

[7] In actuality the 220, 330, and 630-s delays were ~10 s shorter on average, but to avoid confusion when comparing scores obtained during training to those during pseudo mixed-delay testing, we made the delay values the same. The increase in delays presumably occurred because the rats became accustomed to waiting for prolonged periods in the delay area by the end of the training stage.

preceding stage of training, without the confounding effects of practice (Mumby, 2005). The shortest retention interval that could be achieved at this stage was 100 s, in contrast to the 30-s delay during acquisition. It appeared that after the rats received training at longer delays, they became accustomed to waiting in the delay area, and no longer quickly traversed to the test phase. All rats received ten sessions at each delay, administered in blocks of ten such that each rat received ten consecutive sessions with one delay before moving onto a different delay. The type of delay administered first and the sequence of the delays were randomized for each rat. Once a rat completed the pseudo mixed-delay tests, it received ten sessions using a 4-hr delay. Thus, all 4-hr delay tests were conducted last for all rats. The ten sessions at each delay were conducted on different days during a three-week period.

2.2.3.1.6. Probe tests. Following the 4-hr delay tests, probe tests were administered to confirm the rats were not relying on olfactory cues to correctly locate the food reward buried under the novel object on the test phase. Two types of probe tests were conducted: 1) the food reward was omitted on the test (No Reward) and 2) the sample object was baited on the test (Sample-Baited). Two sessions (eight trials) of each type of probe test were performed and compared to two normal test sessions conducted contemporaneously.

2.2.3.2. NOP test. Rats received NOP testing using a 180-s delay. Prior to NOP testing, rats were habituated to the open field arena for ten minutes daily for two consecutive days. Two identical objects were present in the open field arena during habituation. These objects were not used on subsequent experimental trials. Twenty-four hours following the last habituation session, rats received their first trial. A trial consisted of a familiarization phase and a test phase. For the familiarization phase, a rat was placed in the open field arena and allowed to explore two identical sample objects for five minutes. Following a 180-s retention interval, the rat was returned to the arena which then contained a copy of the sample object and a novel object, and the rat was allowed to investigate for five minutes. Objects were counterbalanced between rats such that the sample objects for approximately half of the rats were used as the novel objects for the remaining rats. The side in which the novel object appeared on was counterbalanced between rats and across trials for an individual rat. Each rat received three trials with a 180-s delay and trials were conducted on different days during a two-week period. Different object pairs were used for each trial, but the same object pair was used for all rats on corresponding trials. All three object pairs used in this experiment had been previously screened for preference by a different

group of rats in a nonchoice test. The NOP tests were administered approximately four weeks after the mDNMS tests at the 220-s delay.

Time spent investigating objects was scored using ODLog (Macropod software, version 2.6.1). The rats were considered to be investigating an object if their head was 4 cm away from the object and oriented towards the object, or away from the object at no more than a 45º angle. A rat standing on its hind legs and touching the object with at least one forepaw was also considered to be investigating. Climbing or sitting on top of an object was not considered investigation. The main dependent measure was the investigation ratio. This ratio compares the total object investigation time to the time spent with the novel object during the test phase (Ratio = [$Time_{novel}$/ ($Time_{novel}$ + $Time_{sample}$)]. To determine whether rats' discriminated between the objects, a one-sample *t*-test ($p < .05$) was used to compare mean investigation ratios to chance level of investigation (i.e., a ratio of 0.50). A ratio that was significantly greater than 0.50 indicated the rat spent more time investigating the novel object.

2.2.4. Statistical analyses

Statistical analyses were performed using the *Statistical Program for the Social Sciences* (SPSS) software for Mac (IBM, version 22). The critical threshold for statistically significant results was set at $p < .05$. Eta-squared and Hedge's *g* are reported as measures of effect size.

2.2.4.1. mDNMS task

2.2.4.1.1. Test-retest reliability. The ten sessions from the 220-s delay pseudo-mixed delay testing were used to assess the test-retest reliability of the data. The scores on the 220-s delay sessions were chosen for the reliability analysis because these scores were used to compare performance on the NOP test, which had the same retention delay. A two-way mixed-effects, absolute-agreement ICC was calculated to measure the test-retest reliability of the rats' scores on the 220-s delay sessions.

2.2.4.2. NOP Test

2.2.4.2.1. Test-retest reliability. A two-way mixed-effects, absolute-agreement ICC was calculated to measure the test-retest reliability of the rats' performance on the three 180-s delay NOP trials.

2.2.4.2.2. Correlational analyses. Pearson correlation coefficients were computed to assess the relationship between total time spent investigating the sample objects during the familiarization phase and the average investigation ratios on the test.

2.2.4.3. Comparing performance on the mDNMS task and NOP test. Pearson correlations were computed to assess the relationship between accuracy scores on the mDNMS task and investigation ratios on the NOP test.

2.3. Results

2.3.1. Data Screening

Before conducting any analyses, the data were screened according to the recommended best practices outlined by Kline (2009). The statistical assumptions for one-sample t-tests, ANOVA, and correlation were verified. All scores were standardized in order to detect the presence of outliers. A z-score greater than 3 was used to describe an outlier (Kline, 2009). Standardized scores for each variable did not reveal the presence of any outliers.

The normality of the distribution was assessed for each variable by measuring skewness and kurtosis. Scores were considered normally distributed with a skew less than 3 and a kurtosis less than 10 (Kline, 2009).The distribution of scores was also graphically assessed for normality using a histogram with a normal curve fitted to it. In the current sample, all variables showed acceptable skew and kurtosis, therefore no transformations were applied.

Bivariate scatter plots were investigated to verify the assumptions of linearity and homoscedasticity. A visual inspection of the scatter plots confirmed a linear relationship between variables and confirmed that the homoscedasticity assumption was not violated.

2.3.2. mDNMS Task

One rat failed to reach the performance criterion within the allotted 50 sessions during mDNMS task acquisition. Thus, the results for this rat were excluded from all analyses. Additionally, during the pseudo-mixed delay tests some rats displayed positional biases on at least one of the four test levels (i.e., the rat consistently displaced objects according to their position on the test—right or left—and not whether it was novel). Using a chi-square test of goodness-of-fit it was determined that a significant preference for a particular side that the object appeared on a test level was indicated by a choice of that side on 9 or more of the total 10 trials ($\alpha = .05$). Accordingly, test levels that had a ratio of at least 9 to 1 were excluded from analyses.

This resulted in the exclusion of the following: 1) 10 trials (1 test level) for four rats on the 100-s delay, 2) 20 trials (2 test levels) for one rat and 10 trials for two rats on the 220-s delay, 3) 20 trials for one rat and 10 trials for one rat on the 300-s delay, 4) 10 trials for four rats on the 600-s delay, and 5) 10 trials for five rats on the 4-hr delay.

2.3.2.1. Pre-training. On stage 1, rats reached an accuracy of 92.02% following an average of 6.67 sessions (s = 5). On stage 2, rats reached an accuracy of 83.34% following an average of 6.11 sessions (s = 1.36). Lastly, on stage 3, rats reached an accuracy of 85% following an average of 5.89 sessions (s = 1.83).

2.3.2.2. mDNMS task acquisition. Figure 2.2a depicts DNMS scores on the first and last 5 sessions at each delay during acquisition. Performance during the first training session at the 30-s delay was significantly above chance (M = 67.44%, s = 32.4%). Rats reached a mean accuracy of 84.48% following an average 6.11 sessions (s = 7.24) (excluding criterion sessions). A dependent-samples t-test revealed a statistically significant improvement in scores from the first to the last 5 sessions of acquisition ($t_{(8)}$ = -2.82, p = .01, Hedge's g = -1.26, 95% CI [-2.28, -0.24]). The highest level of accuracy for the rat that failed to reach the performance criterion was 75% by Session 16.

Each time the delay was increased, performance initially declined and then improved with more testing at the new delay. Figure 2.2b depicts the mean number of sessions rats required to reach the performance criterion at increasing delays. Not all rats reached the performance criterion at increasing delays. One rat failed to reach the criterion at the 70 and 220-s delay, four rats failed to reach the criterion at the 90 and 330-s delay, and three rats failed to reach the criterion at the 440 and 630-s delay.

2.3.2.3. Pseudo mixed-delay testing. Figure 2.3 depicts the mean retention curves. The length of the retention intervals increased from 100 to 630 s during this stage of testing, and then to 4-hr in a separate block of trials. The results of a repeated-measures analysis of variance (ANOVA) indicated that performance declined significantly with increases in the retention delay ($F_{(4,32)}$ = 2.86, p = .04, η^2 = 0.36). Follow up t-tests (Bonferroni corrected) revealed a significant difference between the 100-s and 4-hr delay (p = .01). Performance at the 4-hr delay was not significantly above chance ($t_{(8)}$ = 1.61, p = .07, Hedge's g = .76, 95% CI [-0.20, 1.72]). Considering the novelty of the 4-hr delay procedure may have contributed to the near chance score at the 4-hr delay, a separate repeated-measures ANOVA was conducted on scores ranging

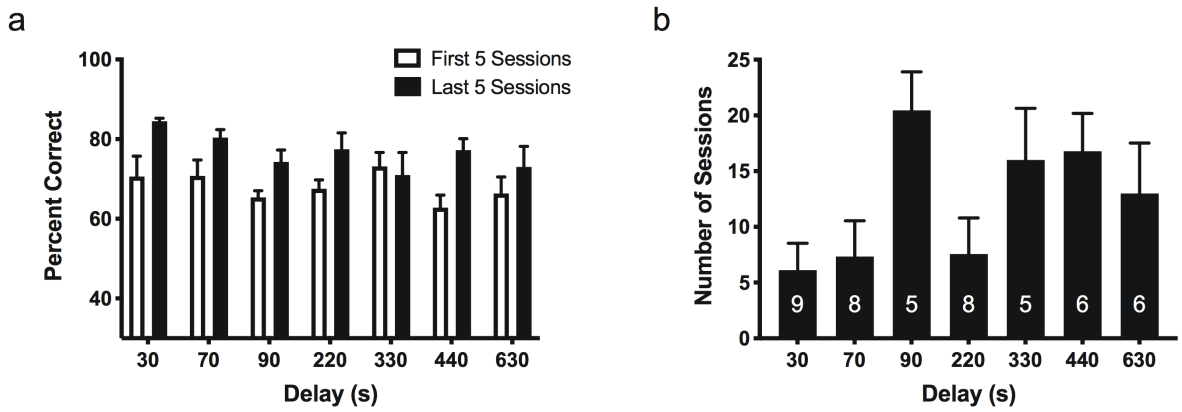

Figure 2.2. Mean scores (± SEM) on the (a) first and last five sessions of the DNMS acquisition phase and (b) mean number of sessions required to reach the performance criterion at each of the seven delays (excluding criterion sessions). The white numerical values on the bars represent the number of rats that attained the performance criterion during that delay.

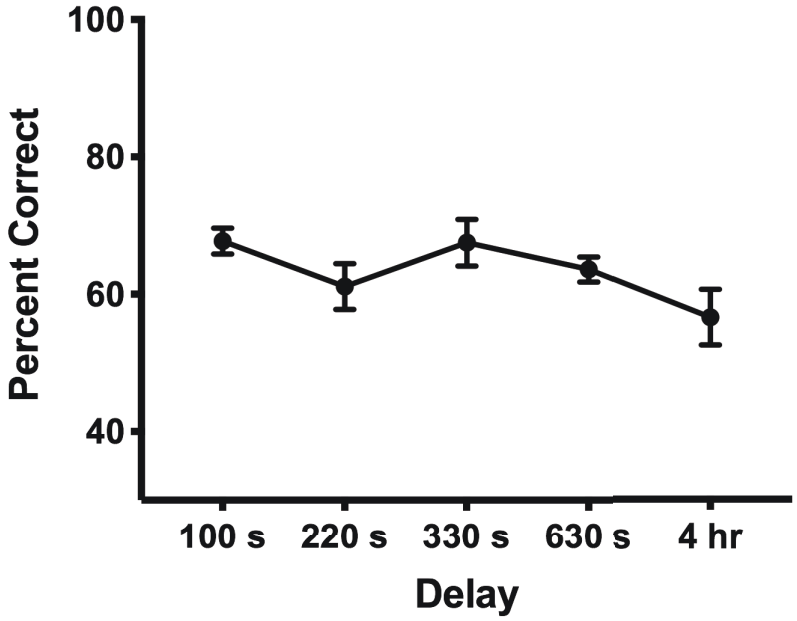

Figure 2.3. Mean scores (± SEM) on the pseudo mixed-delay sessions.

from the 100-630-s delay. This revealed no significant difference in scores ($F_{(3,24)} = 1.49$, $p = .24$, $\eta^2 = 0.19$), suggesting no decline in accuracy with increasing delays.

2.3.2.4. Probe tests. One rat died prior to probe testing, thus the results reported are only for eight rats (Figure 2.4). We compared rats' scores on the probe tests to scores on the normal tests that were administered contemporaneously. Rats' scores were not significantly different from chance on the "No Reward" probe ($t_{(7)} = .27$, $p = .40$, Hedge's $g = .14$, 95% CI [-0.79, 1.07]) and the "Sample-Baited" probe ($t_{(7)} = 1.62$, $p = .08$, Hedge's $g = .82$, 95% CI [-0.15, 1.79]). The scores on the normal tests were also not significantly above chance ($t_{(7)} = 1.18$, $p = .14$, Hedge's $g = .59$, 95% CI [-0.41, 1.59]). In addition, a repeated-measures ANOVA revealed no significant difference between scores on the probe and normal tests ($F_{(2, 14)} = .60$, $p = .56$, $\eta^2 = .09$).

2.3.2.5. Test re-test reliability mDNMS task. One-sample t-tests (one-tailed) revealed that accuracy scores on the 220-s delay were significantly above chance level ($t_{(8)} = 3.38$, $p = .005$, Hedge's $g = 1.59$, 95% CI [0.51, 2.67]). Cohen's κ was computed to assess inter-rater reliability on a random selection of 40% of the trials for each rat. There was excellent agreement between the two raters, $\kappa = .92$, $p < .001$. A two-way mixed-effects, absolute-agreement ICC estimate revealed a good correlation, ICC = .74, 95% CI [.20, .94], $p = .01$. Accordingly, it is estimated that 26% of observed variance is due to random error.

2.3.3. NOP Test

Rats spent on average 85.11 seconds ($s = 21.95$) investigating objects during the 5-min familiarization phase. On the test phase, an average investigation ratio was calculated for each rat, based on the three NOP trials. Ratios were calculated separately for each cumulative minute of the 5-min test. One-sample t-tests (one-tailed) using the first 3-min of the test phase revealed that mean investigation ratios were significantly above chance level ($t_{(8)} = 2.22$, $p = .03$, Hedge's $g = 1.16$, 95% CI [0.15, 2.17]). Thus, on average, rats spent significantly more time investigating the novel objects than sample objects on the test. Scores remained statistically significantly above chance for the remainder of the test, however they were not statistically different from chance during either the first or first two minutes of the test. The time spent investigating objects on the test trials were tested for inter-rater reliability. Thirty-percent of test data were chosen at random for the rater to score. A two-way mixed-effects, absolute-agreement ICC estimate revealed an excellent correlation, ICC = .98, 95% CI [.91, .99], $p < .001$.

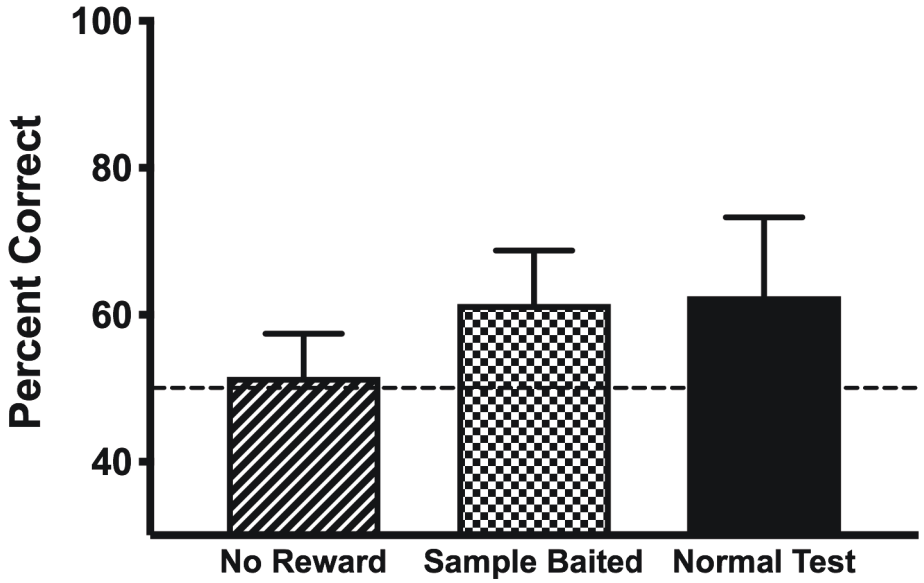

Figure 2.4. Mean scores (± SEM) on the probe and normal test sessions. Dashed line represents chance performance.

2.3.3.1. Test re-test reliability. A two-way mixed-effects, absolute-agreement ICC estimate revealed a poor correlation, ICC = .34, 95% CI [-1.06, .84], p = .23. Accordingly, it is estimated that 66% of observed variance is due to random error.

2.3.3.2. Correlational analyses. There was no significant correlation between total time spent investigating objects during the familiarization phase and investigation ratios (r = -.49, p = .18, 95% CI [-.87, .27]), indicating the magnitude of the novelty preference on the test was unrelated to the amount of sample object investigation during the familiarization phase.

2.3.4. Correlation between scores on the mDNMS task and NOP test

Figure 2.5 shows the results of a correlational analysis of NOP investigation ratios and mDNMS task accuracy scores. There was no significant correlation between average mDNMS accuracy scores and average NOP investigation ratios (r = .29, p = .44, 95% CI = [-.46, .80]), indicating that accuracy to discriminate the familiarity of a previously encountered object on the mDNMS task was not significantly correlated with the magnitude of novel object preference on the NOP test.

2.4. Discussion

One goal of the present experiment was to develop a DNMS procedure that rats could learn more quickly than conventional DNMS tasks, while still performing accurately with retention intervals lasting several minutes. Rats required an average of 24 trials to reach the performance criterion of 84% correct choices on five consecutive sessions (criterion trials not included). By comparison, rats trained using the DNMS procedure described by Mumby and colleagues required on average 174-420 trials to reach a criterion of at least 85% of trials correct on two consecutive sessions (see Table 1 in Appendix A for relevant comparisons between the data reported here and in several previous DNMS studies) (cf. Clark, West, Zola, & Squire, 2001; Duva et al., 1997; Kesner, Bolland, & Dakis, 1993; Kornecook, Kippin, & Pinel, 1999; Mumby et al., 1996; Mumby, Pinel, & Dastur, 1993; Mumby et al., 1990; Mumby, Wood, & Pinel, 1992; Mumby, Mana, Pinel, David, & Banks, 1995; Mumby & Pinel, 1994; Mumby, Pinel, Kornecook, Shen, & Redila, 1995; Wiig & Bilkey, 1995; Wood, Mumby, Pinel, & Phillips, 1993). Rats trained with the Y-maze DNMS task described by Aggleton required on average 130-190 trials to reach a criterion of at least 80% correct trials across five consecutive sessions (Aggleton, Hunt, & Rawlins, 1986; Aggleton, 1985). Moreover, mDNMS task acquisition rate was faster compared to previous studies despite 1) a longer retention delay

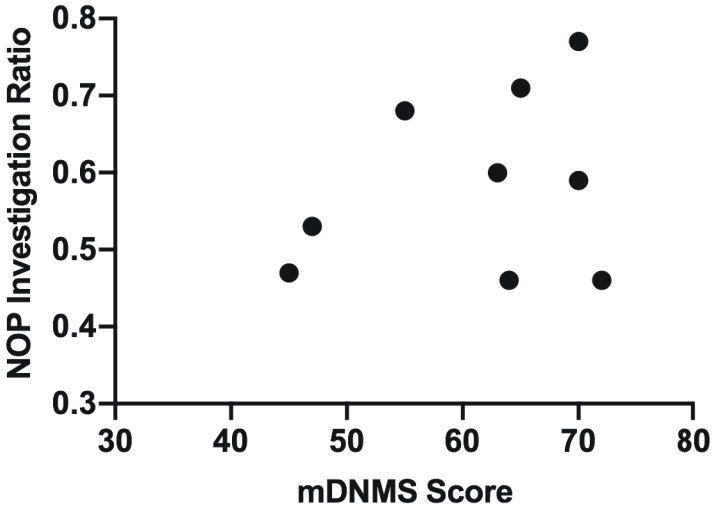

Figure 2.5. Correlation between scores obtained on the mDNMS task and scores on the NOP test. Scores on the two tests were not significantly correlated.

(30 s compared to 0 and 4 s in previous versions), and 2) the presentation of *four* distinct sample objects compared to only one sample object. Thus, rats retained more item information over a longer delay compared to rats in previous studies and were capable of reaching comparable choice accuracy levels in significantly fewer trials. It would appear that rats' performance on the mDNMS task is more robust compared to conventional DNMS tasks. Even in the case of including the pre-training sessions in the calculation for the average number of trials needed to master the task, rats still nevertheless required on average 99 trials. This is still significantly fewer trials compared to the average number of pre-training and training trials required on conventional DNMS tasks (~400 trials on average). In line with our objectives, these findings confirm that the new mDNMS task can be mastered much quicker than conventional DNMS tasks, while requiring little human intervention when administering trials.

During the final stage testing the retention function, rats scored 68%, 61%, 68%, and 64% at delays of 100, 220, 330, and 630 s, respectively (Figure 2.3). These levels of asymptotic performance compare favorably with the asymptotic levels observed at similar retention delays on conventional DNMS tasks (see Table 1 in Appendix A). Thus, compared to conventional DNMS tasks, the modified DNMS task was easier for rats to learn, and they maintained a good level of performance at delays lasting up to 630 s. Accuracy following the 4-hr delay, however, was not significantly above chance levels. These results could be due to potential disruptive effects of the procedure used for the 4-hr delay. Unlike the procedure used for the other delays, during the 4-hr delay the rats were handled, returned to their cage, and transported back to the colony room. This manipulation may have acted as a distraction –a factor known to disrupt performance on DNMS tasks (Hurst & West, 2010; Zola-Morgan & Squire, 1985; Zola-Morgan, Squire, & Amaral, 1989a). Another explanation for the observed impairment is that the rats simply forgot the sample objects over the retention interval. This is plausible considering the rats only briefly encountered each sample object (e.g., between 4 -10 s, 90% of trials). Perhaps this amount of exposure to four distinct objects was not long enough for object information to be held over several hours. Indeed, providing more time to investigate stimulus objects on the sample phase does increase accuracy on DNMS tests (Alexinsky & Chapouthier, 1978; Beck & Kalynchuk, 1992; Nelson & Wasserman, 1978). Regardless of the reason, this finding indicated that we had to modify the procedure to assess object-recognition following delays lasting more than several minutes (the aim of the experiment presented in Chapter 3).

During DNMS acquisition training with progressively longer delays, whenever the delay was increased, there was a transient disruption of choice accuracy followed by a significant recovery (Figure 2.2a). This suggests that rats either gradually learned to avoid distraction for increasing periods of time, or they became more efficient at encoding the attributes of the sample objects as training progressed. In any case, this pattern shows the importance of controlling for the extent of prior training when comparing performance across different retention delays. Providing extensive training at different delays can help rats master other skills that are required for good performance at longer delays that may otherwise mask normal object-recognition abilities (Mumby, 2001).

Several factors are likely to have contributed to making the new mDNMS task relatively easy for rats to learn and perform. On conventional DNMS procedures developed in the 1980s, the experimenter plays an interactive role in administering individual trials. The experimenter can inadvertently distract the rat during testing (e.g., by making sudden movements or sounds). Indeed, a rat that perceives the experimenter as the most interesting thing in the room will pay more attention to the experimenter than to the task at hand. The mDNMS task apparatus has the advantage that the objects can be set up before each trial, and after the rat is placed in the loading cage, the experimenter can quietly leave the room, allowing the rat to "self-administer" trials. Eliminating the presence of the experimenter lessens the potential for distraction.

The lack of a statistically significant correlation between scores on the mDNMS task and NOP test reveal that the two instruments may not be measuring the same underlying construct. A critical feature of any research measurement includes the identification and definition of the variable under investigation. *Construct validity* refers to whether the scores reflect the particular construct (variable) it was designed to measure (Kline, 2009). Major threats to construct validity as described by Kline include: 1) scores that are unreliable and 2) operational definitions that are confounded by other constructs. The findings from the present experiment provide evidence for the mDNMS task being an accurate estimate of object-recognition abilities in rats. First, the rats' scores on the mDNMS task showed good test-retest reliability, indicating that their performance remained stable over time. Second, using *mean percent correct choices* on the test as the operational definition provides a sound indicator of the persistence or accuracy of a rat's memory for the sample object. By requiring the rat to make an instrumental response with which it makes an explicit choice between familiar and unfamiliar objects, and by providing a reward for

accurate choices it teaches the rat the successful strategy it must employ to reach the goal. Ultimately, this decreases the likelihood of some alternative explanation for the observed behaviour on the test. Moreover, by collecting dozens of trials for each individual provides a reliable indication of their ability to discriminate between familiar and unfamiliar objects. Collectively, implementing these elements into the behavioural paradigm increase the accuracy of estimating a rat's ability to discriminate the familiarity of previously encountered objects on the test. Overall, the results from the mDNMS task provide support for it being an effective tool for measuring a rat's object-recognition abilities.

On the NOP test, rats displayed a mean investigation ratio that was significantly above chance, indicating that, on average, the rats' recognized the sample object on the test. The scores, however, showed low test-retest reliability, indicating that rats' novel-object preference scores were not consistent over time. Moreover, the amount of time rats spent investigating objects during the familiarization phase did not predict the magnitude of their novel-object preference on the test. This latter finding is contrary to the assumptions that underlie the manner in which NOP data are usually interpreted: 1) that rats are encoding information about the sample object's features when investigating it during the familiarization phase, and 2) the magnitude of the novel-object preference is a reflection of the persistence or accuracy of a rat's memory for the sample object. Overall, the results from the NOP test suggest that the amount of time a rat spends investigating a novel object compared to a familiar object does not provide an accurate estimate of a rat's object-recognition abilities. Thus, the lack of a statistically significant correlation between scores on the NOP test and mDNMS task in the present experiment likely reflects low internal validity of the NOP test. Consequently, these findings suggest that differences in the magnitude of a novelty-preference on the NOP test should not be uncritically taken to reflect differences in memory ability. It is important to note, however, that both reliability and correlation estimates more closely approximate the population parameter as sample size increases. Thus, when the sample size is low, as was the case in the present experiment, the reliability estimates and correlations may deviate greatly from the population parameter. Thus, the reported reliability estimates and correlations should be interpreted with caution.

Unlike the conventional DNMS tasks on which the reward is delivered only *after* the correct choice has been made, in the present study, the reward was placed under the novel object *prior* to the choice phase. In order to rule out the possibility that rats were locating the food

reward by detecting its odor, we administered probe tests. Rats' performance on both types of probe tests and the normal tests was not significantly different from chance. The low scores on the normal tests make it difficult to interpret the probe test results. If rats were relying on olfactory cues, however, one would predict the accuracy of selecting an object would correspond to the session type, such that rats would exhibit a bias to select novel objects on normal tests and the sample object on the Sample-Baited probe tests. However this was not the case, and in fact, average scores on the Sample-Baited probe tests revealed a tendency for rats to select the novel object first. Additionally, relying on olfactory cues would not be expected to produce delay-dependent changes in performance, as we observed on the pseudo mixed-delay tests between the 100-s and 4-hr delay. Animals can anticipate features of a trial (e.g., the quantity and probability of a reward) and studies have shown that modifying these characteristics on the task can disrupt performance on the test despite intact recognition abilities (Honig & Dodd, 1986). We suspect that introducing these changes to the reward contingency during probe testing disrupted rats' performance on both the normal tests and probe tests, resulting in a decline in accuracy scores. Thus, the decline in task accuracy during probe testing may have reflected the rat's incentive to respond accurately, and not necessarily memory abilities or the ability to detect the odor of the reward. Consequently, we decided to modify the probe test procedure for the experiment in Chapter 3 in an attempt to reduce the disruptive effects of the probe tests.

Scores on pseudo mixed-delay sessions were lower than on the earlier blocked-trial sessions, which used the same delays. We suspect this discrepancy is more likely to reflect an effect of the different testing procedures on *performance* than on memory. As mentioned above, during the blocked-trial sessions with progressively longer delays, there was a transient disruption of choice accuracy during the initial sessions with a new retention delay, followed by a significant recovery to asymptotic levels with continued training at the same delay. This pattern suggests that over several successive trials with a specific retention delay, certain aspects of performance become habitual, and the slight change in procedure that occurs when the delay is lengthened is enough to transiently disrupt them. On the pseudo mixed-delay sessions, the delay changes considerably and on fewer successive sessions, so the disruptive effects are magnified, resulting in lower overall scores during the latter stage of testing. A similar explanation has been previously offered to explain why pigeons' performance on free-operant delayed matching-to-sample is more accurate on trials with long delays than on trials with an unexpected short delay,

if they originally learned the task and received baseline trials with long rather than short delays (Honig & Dodd, 1986; Honig & Wasserman, 1981). This is contrary to what would be expected when measuring working memory, as one would presume accuracy should increase as the working memory demands decrease. These findings suggest that a prospective process (using past experiences to anticipate future responses), and not just memory for trial-specific information may be reflected in task performance (Zentall, 2010). Although the exact mechanisms of this process remain unclear, the findings in the present experiment provoke similar questions about measuring working memory in nonhuman animals; namely, whether a decline in task accuracy as a function of increasing delays truly reflects a loss in working memory capacity (Zentall, 1997). In the future, one way to accommodate for these disruptions in performance could be to provide more than ten sessions at each delay during pseudo mixed-delay testing to give the rats a longer adjustment period to each new delay and to introduce a cue at the beginning of the session to inform the rat of the upcoming delay length.

Six of the nine rats displayed positional biases (e.g., persistently choosing the left object) on at least one of the tests during pseudo-mixed delay testing and subsequent probe tests. This behaviour, if not removed, can impair performance on the task. These positional biases appeared following training on progressively longer delays. Training at longer delays likely contributed to the development of this behaviour because as the delay was increased, so did the demands on memory, as the rat must maintain information about the objects in memory over a longer period of time. Consequently, a rat may resort to this response bias strategy when it fails to remember the sample object on the test (Mumby et al., 1990). Based on the order that the mixed-delay sessions were administered for individual rats, the positional biases for the majority of the rats were found to occur on the ten sessions immediately after the rat had received either testing at the 630-s delay (which it also displayed positional biases on) or training on the 630-s delay (the last training sessions prior to starting pseudo-mixed delay testing). Thus, this response bias strategy formed during sessions when the rat was maintained in the delay area for the longest period of time, and it persisted for several sessions after the delay became shorter. The latter finding reveals that rats persisted to use this strategy for several sessions even after the delay became shorter. Further to the point raised in the previous paragraph, in future experiments whereby rats are tested using retention intervals lasting more than a few minutes, it would be important to provide more sessions at each delay during mixed-delay testing to give rats time to

adjust to each new delay in order to accommodate for the disruptive effects that these changes have on performance.

In summary, the findings from the present experiment demonstrate some of the advantages of using an alternative approach to the conventional DNMS tasks and NOP test to assess rats' object-recognition memory abilities. Compared to conventional DNMS tasks, the mDNMS task was easier for rats to learn and their performance was comparable at similar retention delays. The results from the NOP test and the correlational analysis comparing the two measures suggest that the magnitude of a rat's novel-object preference may not accurately reflect the persistence or accuracy of a rat's memory for the sample object. These results in combination with previous findings (Gaskin et al., 2010; Gervais et al., 2013, 2016), raise concerns regarding the internal validity of NOP test as a measure of object-recognition abilities. Overall, the findings reveal that using an approach that requires rats to make an explicit choice response based on memory, and one that rewards accurate responses on the test provides a less ambiguous interpretation of the status of object-recognition memory in rats.

Chapter 3: Assessing long-term object-recognition memory using the mDNMS task and NOP test

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Abstract

Conventional delayed nonmatching-to-sample (DNMS) tasks cannot be used to assess long-term memory processes because normal rats perform poorly when the retention interval is more than a few minutes. Conversely, normal rats have been shown to exhibit novelty-preferences on the novel-object preference (NOP) test following delays lasting several hours to 24 hours. Moreover, by modifying the familiarization phase, such that rats receive repeated, distributed exposures to the sample object over several consecutive days, rats can display novelty preferences lasting several weeks. We incorporated this procedure that promotes long-lasting memories for sample objects on the mDNMS task, and assessed rats' performance following delays lasting 72 hr, 3 weeks, and ~45 weeks. Rats successfully discriminated between the novel and sample objects on the mDNMS task following all three delays, as evidenced by their above-chance accuracy scores. The latter finding demonstrates that the mDNMS task can be used to assess memory for objects over long delays. The rats were also tested on the NOP test using the same delays, however, they failed to exhibit a significant novel-object preference following both the 72-hr and 3-week delay, and on the ~45-week delay they displayed a significant *sample*-object preference. The divergent findings between rats' performance on both tasks raise concerns regarding the way NOP data are typically interpreted, and reveal that the magnitude of a rat's novel-object preference does not necessarily provide an accurate reflection of its object-recognition abilities.

3.1. Introduction

To investigate the neural processes involved in long-term storage of object information, it is important that an object-recognition task provide a measure of memory for objects learned at widely different time points. The precise mechanisms underlying the processes involved in long-term storage of object information are unknown, as is the exact amount of time this process requires, but evidence suggests that it differs from short-term memory processes and requires *memory consolidation*—the neural process whereby learned information is transferred from a labile state to a long-lasting stable state (Squire & Alvarez, 1995). Moreover, damage made to the medial temporal lobes (MTL) can cause temporally-graded retrograde amnesia whereby memories formed long before the damage remain intact, and more recently formed memories are disrupted (Kim & Fanselow, 1992; Scoville & Milner, 1957; Wiig, Cooper, & Bear, 1996; Zola-Morgan & Squire, 1990). The latter finding has led to theories proposing that the long-term storage of information is only initially dependent on structures within the MTL, but over time information becomes permanently stored in the neocortex (Alvarez & Squire, 1994; Squire, 1992; Squire & Alvarez, 1995).

To date, there have only been a few studies examining rats' memory for objects spanning very long time intervals (e.g., days or weeks), and this is in part due to a lack of suitable tests available. The existing DNMS tasks are not suited to examine long-term object-recognition memory because normal rats perform poorly when the retention interval is more than a few minutes. For this reason, the majority of previous studies have used maximum retention delays of 120 - 300 s (see Table in Appendix A). Some experiments have been conducted using retention intervals lasting 10 min, however, performance on the test declines to near chance accuracy levels (Mumby et al., 1990; Steele & Rawlins, 1989). Accordingly, the existing DNMS tasks are not suitable for assessing memory for objects spanning hours, days, or weeks.

The NOP test on the other hand, can be used to assess rats' object-recognition memory following delays lasting more than several hours. Indeed, normal rats have shown novel-object preferences on the test following delays lasting up to 48 hr (Clark et al., 2000; Winters & Bussey, 2005). Moreover, using a modified procedure can promote long lasting memories for the sample object. By adjusting the familiarization phase on the NOP test, such that rats receive repeated distributed exposures to the sample object over several consecutive days, rats can display novelty preferences after delays as long as 5 weeks (Gaskin et al., 2003; Mumby, Glenn,

et al., 2002; Mumby et al., 2007, 2005). This is important because in order to study the effects of experimental manipulations on long-term object-recognition memory, control animals must show intact recognition abilities at delays lasting at least a few days.

This modified familiarization method has been used in a few experiments to examine the effects of damage made to different MTL structures on rats' memory for objects encountered before the damage. Compared to control rats, rats with hippocampal (Broadbent, Gaskin, Squire, & Clark, 2010; Gaskin et al., 2003) or perirhinal cortex (Mumby, Glenn, et al., 2002) lesions fail to exhibit significant novel-object preferences on tests following learning-to-surgery intervals ranging between 24 hr and 5 weeks. These findings reveal that rats with surgical lesions failed to display novel-object preferences on the test, however, they do not confirm with certainty that this disruption reflects an effect of the treatment on object-recognition abilities. When a rat exhibits a novel-object preference after some experimental manipulation, it is clear that the treatment failed to disrupt object-recognition memory. However, when a rat fails to exhibit a novel-object preference after some treatment, it is difficult to confirm with the same level of certainty that the rat has failed to recognize the sample object. The treatment, for example, may have altered the behavioural expression of this novelty preference for reasons unrelated to failures in object-recognition memory (e.g., increase neophobia). Alternatively, the treatment may have disrupted some other mnemonic process. Given the nature of the NOP test, whereby the rat spontaneously explores objects in an open field, the rat is learning information not only about the features of the objects, but also about their location relative to the surrounding context. Accordingly, on the test phase, a rat may rely on both spatial and nonspatial information to successfully discriminate between the sample and novel object. Consequently, when a rat fails to display a novel-object preference following some treatment, it could reflect a disruption in memory for the: 1) sample object, 2) context (e.g., testing room), or 3) specific location of the sample object relative to cues within the apparatus (including the second copy of the sample object). This raises concerns about potential confounds that are introduced when trying to estimate a rat's memory for a previously encountered object when using the NOP test.

The number of potential reasons for why a rat fails to display a novelty preference on the test is complicated by the fact that the NOP test does not involve a goal, and thus the rat is not required to make an explicit choice response based on memory. Indeed, the lack of an instrumental response complicates the interpretation of the behaviour because it fails to limit the

number of alternative explanations, besides an object-recognition memory impairment, for the observed behaviour on the test. Conversely, using a task, such as the mDNMS task, whereby the rat makes an unambiguous instrumental response, receives rewards for accurate choices, and receives dozens of trials, provides a more straightforward interpretation of behaviour in relation to the specific construct under investigation: object-recognition memory.

The primary goal of the present experiment was to determine whether the mDNMS task can be used to assess rats' memory for objects following long retention intervals (72 hr, 3 weeks, and ~45 weeks). A second goal was to test the same rats on the NOP test using the same delays and compare performance on both tasks. In order to assess object-recognition memory following long-retention intervals, we used the procedure that we previously found promotes long-lasting memories for sample objects on the NOP test by providing repeated exposures to various sample objects over several consecutive days. For the ~45-week delay, rats were familiarized to sample objects for both the NOP test and mDNMS task starting in periadolescence (7 weeks old) during an environmental enrichment program.[8] The rats were placed together in an apparatus similar to the mDNMS task apparatus and encountered different sample objects for five hours per day over several weeks. Once the rats were ~50 weeks old, after they received mDNMS training, their memory for those sample objects was tested using both the mDNMS task and NOP test. For the 72-hr and 3-week delay rats were familiarized to sample objects for both the mDNMS task and NOP test using a third apparatus—a circular-track. This apparatus allowed for the presentation of multiple objects concurrently for extended periods of time (Piterkin, Cole, Cossette, Gaskin, & Mumby, 2008). Afterwards, we tested rats' memory for the NOP objects by presenting them in an open field arena and for the mDNMS objects by presenting them on the test side of the large multi-level mDNMS apparatus.

To confirm that the rats accurately discriminated between objects following a less taxing retention interval on both the mDNMS task and NOP test, we also administered tests using a short delay while collecting data for each long delay. The short delay tests were conducted using a similar procedure as in the experiment presented in Chapter 2.

[8] The program entailed placing a group of rats (starting on post-natal day 28) in a large multi-level environment and exposing them to a variety of events over a 14-week period. For details see Section 3.2.1.

3.2. Materials and Method

3.2.1. Subjects

The subjects were 11 male Long-Evans rats (Charles River, Kingston, ON), weighing 450-550 g at the start of mDNMS training (~22 weeks old). The rats were pair-housed in polypropylene cages (48 × 25 × 20 cm) in a colony room maintained under a reverse 12:12 light-dark cycle, with light onset at 8:00 p.m. The rats had continuous access to water and each received a daily ration of ~25 g of rat chow (Charles River Rodent Animal Diet, no. 5075) in the late afternoon, after behavioral testing was finished for the day. Prior to the start of mDNMS task training, rats received 14 weeks of environmental enrichment starting on post-natal day 28. Environmental enrichment entailed placing all 11 rats in a large apparatus, similar to the one used on the mDNMS task, for five hr/day, five days/week. During environmental enrichment, the rats were familiarized to sample objects for extended periods of time for the ~45-week delay and were exposed to different events as part of an unrelated experiment. Specifically, rats had the opportunity to socialize, forage for novel foods, and on occasion encounter aversive stimuli (e.g., a lithium chloride injection following the ingestion of a novel food and a collar infused with cat odor). Following enrichment the rats were used in a series of brief unrelated experiments involving exposure to aversive stimuli (e.g., receiving a foot-shock in a conditioning chamber or being placed in a water maze). The training histories were identical for all rats in the present experiment. All procedures were approved by the Concordia University Animal Care and Use Committee, and were in accordance with the guidelines of the Canadian Council on Animal Care.

3.2.2. Apparatuses

3.2.2.1. Enrichment apparatus. The apparatus dimensions were the same as the mDNMS task apparatus (152 × 145 × 86 cm). The design was similar such that rats entered the environment via a loading cage placed on top of the apparatus, and there were five levels, the top four of which had divider walls. The floor substrate varied across levels and consisted of wood chips, sand, or wood pellets.

3.2.2.2. mDNMS task. A large multi-level environment (152 × 145 × 86 cm) was used to test the rats (see Chapter 2 Figure 2.1). The apparatus was a modified, freestanding steel cage rack, enclosed on three sides by wire mesh, with a removable, clear acrylic front panel. The apparatus had five levels, each covered with woodchip. The top four levels were divided into two

equal halves by a plastic barrier wall, and the bottom level remained undivided. A loading cage (58 × 37 × 20 cm) was placed on the top left side of the apparatus. A rat entered the apparatus via a hole in the bottom of the loading cage that was placed over a passageway leading to the top level of the apparatus. Rats traversed the different levels via wire mesh passageways located on both sides of the apparatus. The design of the apparatus was such that a rat had to climb down the passageways on the left side of the apparatus in order to gain access to the right side, which it then could ascend from level to level. The top four divided levels contained plastic rectangular platforms (30 × 12 × 1 cm) each with a recessed food well (2 cm in depth), over which stimulus objects could be placed. One platform was placed on each level on the left side of the apparatus, and on the right side of the apparatus, two platforms were placed on each level with the food wells 9 cm apart. All platforms were positioned near the middle barrier wall, in line with the passageway that provided access to the level. The room contained dim lights (40 lx) and a video camera was positioned in front of the apparatus in order for the experimenter to watch the session on a TV monitor in an adjacent room.

3.2.2.3. NOP test. The apparatus for the NOP test phase was an open-field arena (60 × 70 × 70 cm), constructed of grey PVC plastic. The floor of the arena consisted of a stainless-steel tray covered with woodchips. The floor was removable via a slot at the bottom of one wall to facilitate changing the woodchips between each trial. The testing room contained dim lights (14 lx) and a videocamera was positioned over the arena to record the sessions for later analysis.

3.2.2.4. Circular-track apparatus. Figure 3.1 illustrates the apparatus used for the sample phase on the 72-hr and 3-week delay. The floor of the track was 30 cm wide, and formed a circle with an outside diameter of 270 cm. The floor of the track was covered with wood chips. The inside and outside walls of the track extended from the floor to a height of 40 cm, and both walls had a slight concave curvature. Modular divider-walls separated the track into 8 equally sized compartments. One compartment was used as a “start location” where the rat was placed at the beginning of each trial, and the remaining compartments were either used to present a single object (locations depicted in Figure 3.1) or empty. A rat could circulate the track in either direction via small doors located at the based of the divider walls. The testing room contained dim lights (30 lx) and a video camera was positioned above the apparatus to record the sessions for later analysis.

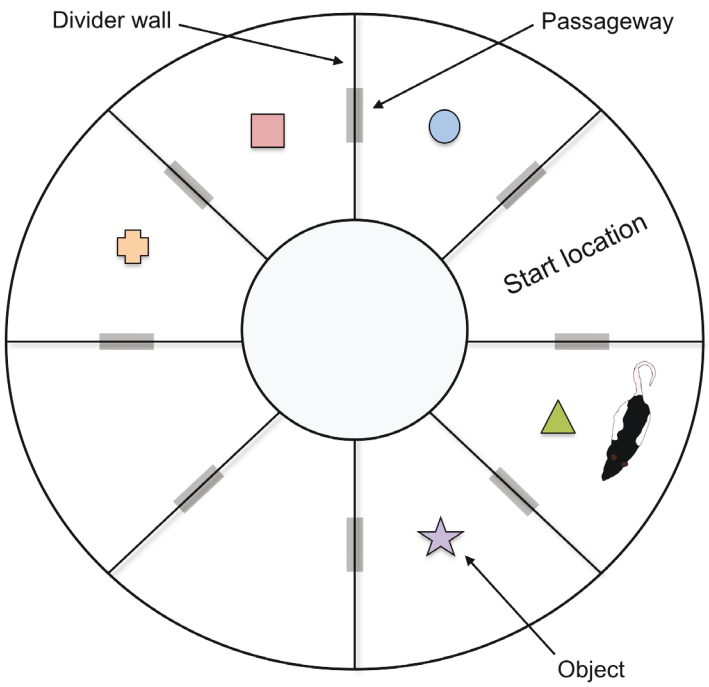

Figure 3.1. Circular-track apparatus depicting a trial for the familiarization phase for the 72-hr and 3-week delay mDNMS and NOP tests. The apparatus was divided into 8 equally-sized compartments with one start location compartment and seven object compartments, five of which were used to present objects, and two remained empty. A rat could circulate the track in either direction via passageways located at the based of each divider wall.

3.2.2.5. Stimulus objects. A total of 384 different mDNMS objects were used as stimuli for the mDNMS task. Objects were made of plastic, metal, glass, or glazed ceramic, and ranged in size from 4 to 18 cm in height, and 4 to 13 cm in width. Each object was large enough to cover the food well but light enough to be easily displaced by a rat. There were two copies of each mDNMS task object –one for the sample phase and one for the test. The objects used for the sample phase for the 72-hr, 3- and ~45-week delay had a small container (2.5 cm high) that was attached to the bottom of the object with epoxy. The objects were then fixed in place by screwing the containers into inverted lids that were attached to a ceramic tile (10 × 10 cm), which was then buried under 2.5 cm of wood chips to stabilize it. The objects were cleaned using a 70% ethanol solution after every trial on which they were used. At the end of each day the mDNMS task objects were cleaned using a diluted bleach solution (1:20 concentration ratio).

A separate pool of 40 objects was used for the NOP test. Objects for the NOP test were made of plastic, metal, glass, or glazed ceramic, and ranged in size from 7 to 18 cm in height, and 5 to 13 cm in width. A small glass jar (6 cm high) was attached to the bottom of each object with epoxy. For the 180-s delay, the NOP trials were conducted in the open field arena, in which case there were three copies of each object—two for the familiarization phase and one for the test. The objects could be fixed in place by screwing the jars into inverted lids that were attached to the stainless-steel tray in the open field arena (positioned 27 cm from opposing corners). For the 72 h, 3- and ~45-week delay, there were only two copies of each NOP object –one for the familiarization phase and one for the sample on the test phase. The objects could be fixed in place by screwing the jars into inverted lids that were attached to removable platforms (10 × 10 cm) that were placed in either the enriched environment (sample phase for the ~45-week delay) or the circular-track (sample phase for the 72-hr and 3-week delay).

The test phase for each delay was conducted in the open field arena, in which case the jar on the bottom of each object was screwed into one of the two inverted lids that were attached to the stainless-steel tray in the open field arena. The objects were cleaned after every trial on which they were used, by wiping with a damp paper towel. At the end of each day the NOP objects were cleaned using a 70% ethanol solution.

3.2.3. Chronology of experiment

Figure 3.2 depicts a timeline for the experiment. Rats were first familiarized to objects for the mDNMS task and NOP test ~45-week delay. Next, they received mDNMS task training,

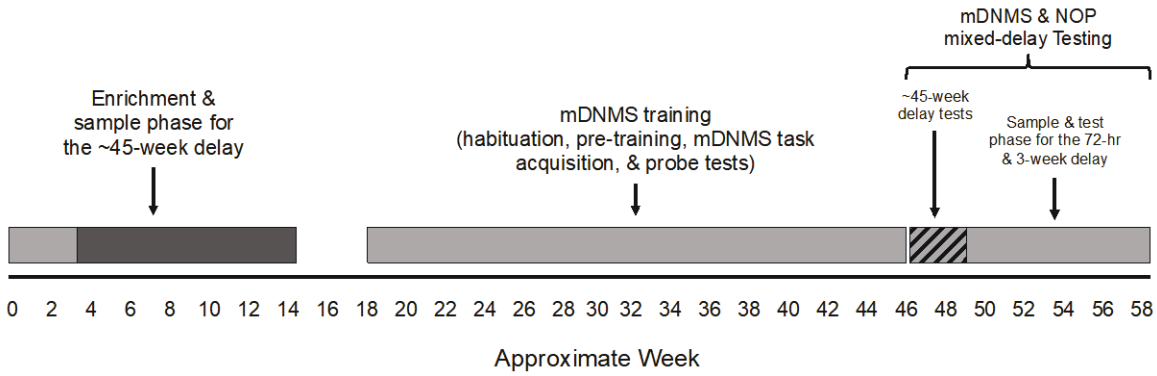

Figure 3.2. Timeline depicting the sequence and average duration of each phase of the procedure for both the mDNMS task and NOP test. Gray bars represent length of the phase. Shaded portion of the enrichment phase bar depicts the object familiarization period for the ~45-week delay. The gap between enrichment and mDNMS training reflects the period when the rats were used in an unrelated experiment.

followed by probe tests. Afterwards, rats received the test phase for the ~45-week delay using both the mDNMS task and NOP test, followed by testing using a 72-hr and 3-week delay.

Throughout mixed-delay testing, rats also received tests on the mDNMS task using an 80-s delay and on the NOP test using a180-s delay.

3.2.4. Behavioral procedures

3.2.4.1. ~45-week delay familiarization phase for the mDNMS task and NOP test.

During the last 12 weeks of environmental enrichment, rats received the sample phase for the ~45-week delay by exposing them to a total of 48 objects in the enriched environment. To ensure rats spent an approximately equal time investigating each object, the objects were staggered such that only four objects were placed in the environment at a time. Over the course of 10 days, rats were exposed to a total of eight different objects (two sets of four objects on alternating days). On Day 1, 3, 5, 7, and 9 rats encountered Objects 1–4 and on Day 2, 4, 6, 8, and 10 rats encountered Objects 5–8. On each day, rats were exposed to the set of objects for five hours. This procedure was repeated five times throughout the 12-week period, using different object sets each time. The objects were fastened to the floor on the left side of the apparatus; one on each of the top four levels placed in the same spot where sample objects appear on the mDNMS task sample phase. The level in which an object appeared was varied each day, such that an object appeared at least once on each level. Twenty of these objects later served as the sample objects on the test phase for the mDNMS task and five served as sample objects on the NOP test. The same objects served as the sample for all rats. The remaining 23 sample objects were not used.

3.2.4.2. mDNMS task. Following the environmental enrichment period, rats were trained on the mDNMS task. There were three stages: 1) habituation, 2) pre-training, and 3) mDNMS task acquisition.

3.2.4.2.1. Habituation. The rats were handled for ~10 minutes daily for one week before they were habituated to the apparatus. The goal of habituation was to have rats complete an entire circuit of the apparatus (start on the top left level and finish on the top right level), with relatively little hesitation. Rats received one habituation session per day. On the first 20 sessions, all 10 rats were placed in the apparatus for 30 minutes with no stimulus objects present, and ~20 Cheerios (1.8 g, General Mills) were placed on each level near and inside the food wells. On the final two habituation sessions, the rats were placed in the apparatus in pairs for 5 minutes, and

only 5 Cheerios were placed inside each food well. By this point, each rat was consistently eating Cheerios from each food well and reliably completing the entire circuit. During this stage and subsequent stages, the experimenter left the room after placing the rats in the apparatus and watched the session on a TV monitor in an adjacent room.

3.2.4.2.2. Pre-training. Pre-training consisted of three stages; stage 1, 2, and 3 (refer to Figure 2.1a-c). The rats were now tested individually and they were introduced to stimulus objects. The purpose of the pre-training stages was to gradually familiarize the rats to the procedural aspects of the task (e.g., learn to displace objects from over food wells and to dig for a buried Cheerio placed in food wells) and to teach them that the visual/tactile object features were key to predicting food location. Pre-training stage 1, 2, and 3 differed in the number of distinct sample objects that were presented to the rat: one, two or three, respectively. The rat had to reach a performance criterion at each stage before advancing to the next stage.

On each stage, a rat received one session per day, which consisted of two phases: a sample phase and a test phase. On the sample phase, the rat descended the left side of the apparatus and encountered either four copies of one sample object (stage 1, see Figure 2.1a), two copies of two different sample objects (stage 2, see Figure 2.1b), or three different sample objects –two copies of one object on the top two levels, and two distinct sample objects on the bottom two levels (stage 3, see Figure 2.1c). One Cheerio was placed in each food well to encourage the rat to approach and investigate the sample objects. On the test phase, the rat ascended each level on the right side of the apparatus encountering a different novel object paired with a copy of the sample object and one Cheerio was placed in the food well under each novel object. Thus, the test phase consisted of four separate 'trials', one for each level. On stage 2 and 3 the sample objects on the test phase were presented in the same order that the rat had encountered them on the sample phase (i.e., the first sample object appeared on the first test level). The sample and test phase were separated by a short retention interval in which the rat spent traversing the bottom level of the apparatus. On the first few stage 1 sessions, the objects only partially covered the food well to encourage timid rats to displace objects. As sessions continued, the objects were gradually positioned to cover the entire food well. During stage 2, the Cheerios on the sample and test phase were gradually buried beneath woodchip until the food well was entirely filled to the top (2 cm deep) and rats were consistently digging for the Cheerio. Burying the Cheerio was done in an attempt to reduce the likelihood that a rat would rely on

olfactory cues to locate the reward. Moreover, by making the rat dig for the Cheerio we increased both the delay and amount of effort necessary to retrieve the Cheerio from beneath the novel object.

A correct choice on a test trial was defined as the rat either displacing the novel object before displacing the sample object, or only displacing the novel object. An incorrect choice was defined as the rat only displacing the sample object, or displacing the sample object before the novel object. If a rat did not displace either object on a particular test, it was considered a non-trial. For a particular rat, we began recording its accuracy on the test phase once all objects fully covered the food well. Different sample and novel objects were used on each session. On stage 1 a total of 15 different object sets were used, each containing 8 copies of one sample object and 4 unique novel objects. After 15 sessions, rats re-encountered the objects again in the same sequence, starting with the first object set. On stage 2 and 3 four new object sets were introduced –each containing four copies of two distinct sample objects and four unique novel objects. These objects sets were used in combination with the stage 1 sets. The location of the novel object on the test phase was counterbalanced in a pseudorandom order.

After the rat completed the final test, the experimenter entered the room and removed the rat from the top right side of the apparatus. Between each rat, the woodchip on every level was redistributed to spread any potential odor cues left by a previous rat and each object platform was cleaned using a 70% ethanol solution. A rat advanced to the next pre-training stage once it reached a performance criterion of at least 80% of trials correct on five consecutive sessions (i.e., at least 16 correct trials out of 20 trials). A rat was given a maximum of 50 sessions at each stage to reach the performance criterion.

3.2.4.2.3. mDNMS task acquisition. During the final training stage, rats encountered four distinct sample objects, one on each of the divided levels of the sample phase (see Figure 2.1d). Thus, this stage was similar to conventional DNMS tasks in that each sample object was encountered only once during the sample phase and was subsequently paired with a unique novel object for the test phase. Similar to pre-training, a session consisted of a sample and test phase. On the sample phase, a rat descended the apparatus to familiarize itself with four distinct sample objects, encountering a different one on each level. One Cheerio was buried in the food well under each sample object. During the test phase, a copy of each sample object was presented

next to a novel object. A Cheerio was buried under the novel object on each test level. Each session consisted of four trials, as there were four distinct sample objects in the apparatus.

From this point forward a new collection of object sets was used. The objects changed on each session, however, the same objects served as the sample objects and novel objects for all rats. Once a particular object was used on a session, it was not used again until all of the objects in each set were used. This resulted in a particular object re-occurring approximately every 20 sessions. Moreover, an object that served as a sample the first time a rat encountered it, served as a novel the next time it was encountered (and vice versa). The sample and novel object on each trial were paired based on similarities in size, weight, and material. The location of the novel object on each test was counterbalanced in a pseudorandom order. A rat was required to reach a performance criterion of at least 80% of trials correct on five consecutive sessions (16 trials correct out of 20). The average delay between the sample and test phase was 69 s (s = 21.87). A rat was given a maximum of 50 sessions at this stage to reach the performance criterion. Rats received one session per day and were tested no fewer than four days per week. The dependent measures were mean percent correct choices and mean number of sessions required to reach the performance criterion.

3.2.4.3. Probe tests. Following mDNMS task acquisition we confirmed that the rats were not relying on olfactory cues to correctly locate the food reward buried underneath the novel object on the test phase. The same two types of probe tests were administered ("No Reward" and "Sample Baited"), except now each type of probe test was administered *within* normal test sessions, rather than simultaneously in one session like in the experiment presented in Chapter 2, in an attempt to reduce the disruptive effects of probe tests. All rats received 10 trials on each probe test, and the tests occurred on consecutive sessions.

3.2.4.4. mDNMS and NOP mixed-delay testing

3.2.4.4.1. mDNMS ~45-week delay test phase. Rats received 20 trials on the ~45-week delay, which occurred after mDNMS task acquisition. The ~45-week delay tests were administered in the mDNMS apparatus and were conducted concurrently with short delay trials, such that on each session, a rat received two tests using a ~45-week delay and two tests using a short delay. To administer the short delay trials, the apparatus was setup in the standard way but with only two sample objects on the left side of the apparatus—each randomly placed on one level—and two tests on the test side. The two remaining test levels were setup with objects for

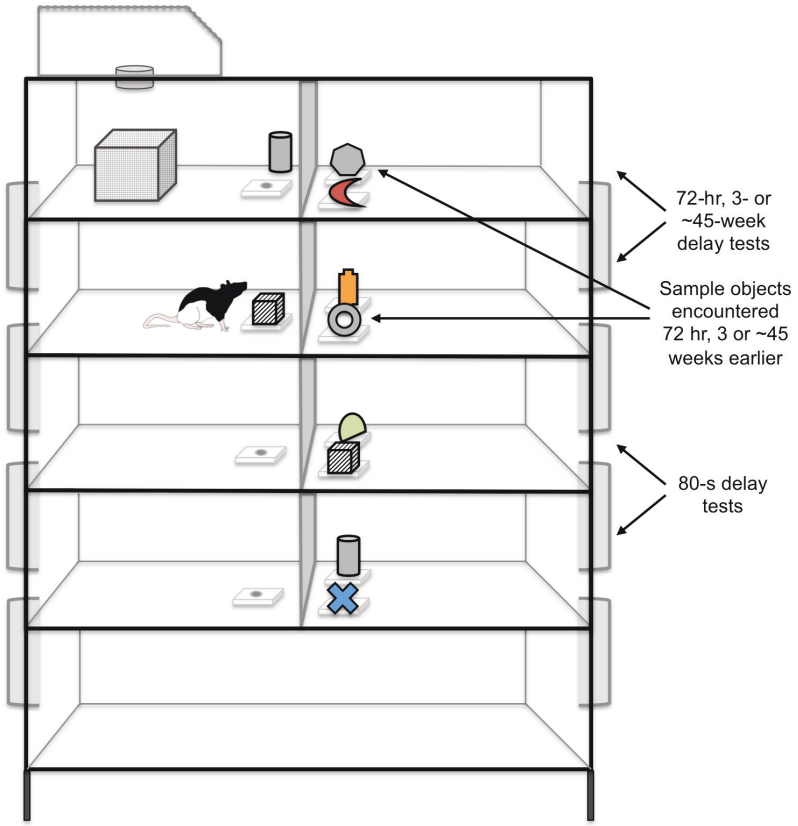

Figure 3.3. Diagram of one mDNMS test session for the mixed-delay testing. On the left side of the apparatus, two levels contained sample objects for the 80-s delay and two remained empty—representing the sample phase that the rat had already received for either the 72-hr, 3- or ~45-week delay. The level that the sample objects for the 80-s delay appeared on was pseudo-randomized across sessions. On the test side of the apparatus, two tests were setup for the 80-s delay (objects the rat had encountered within that session) and two tests were setup for either the 72-hr, 3- or ~45-week delay, depending on the type of delay that was administered on that particular session. On the test phase, a sample object that the rat had encountered previously (80 s, 72 hr, 3 weeks, or ~45 weeks earlier) was paired with a novel object. One Cheerio was placed under each novel object. The number of test trials on each particular delay was equally distributed across the test levels.

the ~45-week delay tests (see Figure 3.3). The short retention interval was on average 80 s (*s* = 58.11). The test location was counterbalanced across sessions, such that a test for each delay occurred equally often on each of the four test levels. The location of the novel object was counterbalanced across trials such that it occurred equally often on the left and right side. The object sets used for the short-delays were presented in a similar fashion as in the experiment presented in Chapter 2, such that once a particular object was used on a session, it was not used again until all of the objects in each set were used. Moreover, an object that served as a sample the first time a rat encountered it, served as a novel the next time it was encountered (and vice versa). Conversely, none of the novel objects used for the ~45-week delay tests had been previously encountered by the rats, and thus were trial-unique.

3.2.4.4.2. NOP ~45-week delay test phase. Rats received five NOP trials on the ~45-week delay, which were conducted in an open field arena. Prior to NOP testing, rats were habituated to the open field arena for ten minutes daily for two consecutive days. Two identical objects were present in the open field arena during habituation. These objects were not used on subsequent experimental trials. Twenty-four hours following the last habituation session, rats received their first test. The test procedure involved placing the rat in the open field arena with a copy of the sample object and a novel object for five minutes. Different object pairs were used for each trial, but the same object pair was used for all rats on corresponding trials. The side in which the novel object appeared on was counterbalanced between rats and across trials for an individual rat. The NOP tests were administered contemporaneously with the ~45-week delay mDNMS tests. Testing on both tasks never occurred on the same day for any individual rat. The type of task (NOP or mDNMS) that a rat started with was pseudo-counterbalanced across rats, such that approximately half of the rats started with a trial on the mDNMS task and the other half started with a NOP trial.

Time spent investigating objects was scored using ODLog (Macropod software, version 2.6.1). The rats were considered to be investigating an object if their head was 4 cm away from the object and oriented towards the object, or away from the object at no more than a 45º angle. A rat standing on its hind legs and touching the object with at least one forepaw was also considered to be investigating. Climbing or sitting on top of an object was not considered investigation. The main dependent measure was the investigation ratio. This ratio compares the total object investigation time to the time spent with the novel object during the test phase (Ratio

= [$Time_{novel}$/ ($Time_{novel}$ + $Time_{sample}$)]. To determine whether rats' discriminated between the objects, a one-sample *t*-test ($p < .05$) was used to compare mean investigation ratios to chance level of investigation (i.e., a ratio of 0.50). A ratio that was significantly greater than 0.50 indicated the rat spent more time investigating the novel object.

3.2.4.4.3. Sample phase for the 72-hr and 3-week delays. Rats were familiarized to objects for both the mDNMS task and NOP test using the circular-track apparatus. The sample phase for the 72-hr and 3-week delay was administered concurrently in the circular-track apparatus. Over four consecutive days, rats were familiarized to ten distinct sample objects. On Day 1 and 3 rats encountered Objects 1–5 and on Day 2 and 4 they encountered Objects 6–10. On each day, a rat received three distributed 10-min trials, each separated by one hr, and the objects changed location in a clockwise fashion across trials. Of the ten objects, eight were designated mDNMS sample objects and two were designated NOP sample objects. Moreover, half of the respective task objects were used for the 72-hr delay and the remaining half for the 3-week delay (i.e., four mDNMS objects and one NOP object for each delay). This procedure was repeated four times, using different objects each time. Thus, rats encountered a total of 50 objects –40 mDNMS objects (20 objects for each delay) and 10 NOP objects (5 objects for each delay). The objects were pseudo-counterbalanced between rats, such that the sample objects for approximately half of the rats were used as the novel objects for the remaining rats. All of the sample and novel objects used for the 72-hr and 3-week delay were trial-unique, such that a rat had never encountered them (with the exception of some mDNMS sample objects that had been used once as a novel object on the ~45-week delay tests). The operational definition for *object investigation* was the same as described in Section 3.2.4.4.2. After placing the rat in the apparatus, the experimenter left the room and watched the session on a TV monitor in an adjacent room.

3.2.4.4.4. mDNMS 72-hr and 3-week delay test phase. Rats received 20 trials on both the 72-hr and 3-week delay in a mixed fashion. Similarly to the ~45-week delay tests, the tests were administered two at a time concurrently with short-delay trials, such that on the test phase there were two tests setup for a 80-s delay and two tests set up for either a 72-hr or 3-week delay (see Figure 3.1).

3.2.4.4.5. NOP 72-hr and 3-week delay test phase. Rats received five trials on both the 72-hr and 3-week delay in a mixed fashion. The NOP tests were administered

contemporaneously with the 72-hr and 3-week delay mDNMS tests. Testing on both tasks never occurred on the same day for any individual rat. The type of task (NOP or mDNMS) that a rat started with was pseudo-counterbalanced across rats, such that approximately half of the rats started with a trial on the mDNMS task and the other half started with a NOP trial.

3.2.4.4.6. NOP 180-s delay testing. Rats received five NOP trials using a 180-s delay throughout mixed-delay testing. While collecting data for the ~45-week delay, two 180-s delay NOP trials were administered and the remaining three trials were administered during testing using the 72-hr and 3-week delays.

3.2.5. Statistical analyses

Statistical analyses were performed using the *Statistical Program for the Social Sciences* (SPSS) software for Mac (IBM, version 22). The critical threshold for statistically significant results was set at $p < .05$. Eta-squared and Hedge's *g* are reported as measures of effect size.

3.3. Results

3.3.1. Data Screening

Before conducting any analyses, the data were screened according to the recommended best practices outlined by Kline (2009), and the statistical assumptions for each type of analysis were verified. All scores were standardized in order to detect the presence of outliers. A z-score greater than 3 was used to describe an outlier (Kline, 2009). Standardized scores for each variable did not reveal the presence of any outliers.

The normality of the distribution was assessed for each variable by measuring skewness and kurtosis. Scores were considered normally distributed with a skew less than 3 and a kurtosis less than 10 (Kline, 2009).The distribution of scores was also graphically assessed for normality using a histogram with a normal curve fitted to it. In the current sample, all variables showed acceptable skew and kurtosis, therefore no transformations were applied.

Bivariate scatter plots were investigated to verify the assumptions of linearity and homoscedasticity. A visual inspection of the scatter plots confirmed a linear relationship between variables and confirmed that the homoscedasticity assumption was not violated. Thus, the data met the assumptions for calculating Pearson correlation coefficients.

3.3.2. mDNMS task

During pre-training stage 1, three rats had positional biases that could not be removed (i.e., the rat consistently displaced objects according to their location on the test—right or left—

and not whether it was novel or familiar). Additionally, one rat failed to reach the performance criterion within the allotted 50 sessions during mDNMS task acquisition. Thus, the results for these four rats were excluded from all analyses. Furthermore, due to human error, four trials for one rat and two trials for another rat were excluded from the 72-hr delay analyses, and one trial for two rats was excluded from the 3-week delay analyses.

3.3.2.1. Pre-training. During stage 1, 2, and 3, rats reached an accuracy of 80.26%, 80.83%, and 80% respectively, following an average of 15.43 (s = 10.29) sessions in stage 1, 15.71 sessions (s = 13.96) in stage 2, and 8 sessions (s = 5.20) in stage 3.

3.3.2.2. mDNMS task acquisition. Performance during the first training session was significantly above chance (M = 71.43%, s = 26.73%). Rats reached a mean accuracy of 81.43% following an average of 19.86 sessions (s = 14.92). A dependent-samples t-test revealed a statistically significant improvement in scores from the first to the last five sessions of acquisition ($t_{(6)}$ = -4.96, p = .003, Hedge's g = -3.11, 95% CI [-4.74, -1.48]).

3.3.2.3. Probe tests. We compared rats' scores on the probe tests to scores obtained on the normal tests that were administered concurrently (Figure 3.4). Rats' scores were significantly above chance on the "No Reward" probe ($t_{(6)}$ = 2.76, p = .02, Hedge's g = 1.47, 95% CI [0.27, 2.67]), the "Sample Baited" probe ($t_{(6)}$ = 1.99, p = .04, Hedge's g = 1.06, 95% CI [-.07, 2.19]), and the normal tests administered at the same time as the probe tests ($t_{(6)}$ = 2.25, p = .03, Hedge's g = 1.20, 95% CI [.05, 2.35]). A One-way ANOVA revealed no significant difference between scores on the probe and normal tests ($F_{(2, 18)}$ = .89, p > .05, η^2 = .09).

3.3.2.4. mDNMS task mixed-delay testing. Due to human error, four trials for one rat and two trials for another rat were excluded from the 72-hr delay analyses, and one trial for two rats was excluded from the 3-week delay analyses.

Rats spent on average 7.17 s (s = 1.82), 90.61 s (s = 16.51), and 99.89 s (s = 28.59) investigating objects during the sample phase for the 80-s, 72-hr, and 3-week delay, respectively (see Figure 3.5a).

Figure 3.6a depicts rats' performance on the mDNMS tests at each delay. Accuracy scores were significantly above chance at the 80-s delay ($t_{(6)}$ = 4.64, p = .004, Hedge's g = 2.47, 95% CI [1.03, 3.91]), the 72-hr delay ($t_{(6)}$ = 5.41, p = .002, Hedge's g = 2.90, 95% CI [1.34, 4.46]), the 3-week delay ($t_{(6)}$ = 12.88, p < .001, Hedge's g = 6.99, 95% CI [4.0, 9.98]), and the ~45-week delay ($t_{(6)}$ = 6.22, p = .001, Hedge's g = 3.33, 95% CI [1.64, 5.02]).

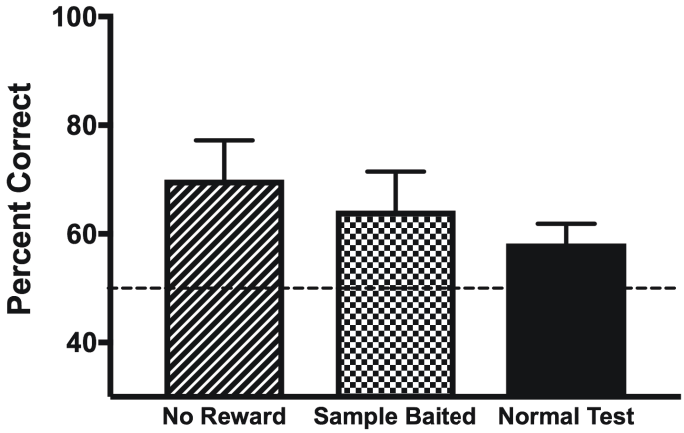

Figure 3.4. Average scores (± SEM) on the probe and normal test trials. Dashed line represents chance performance.

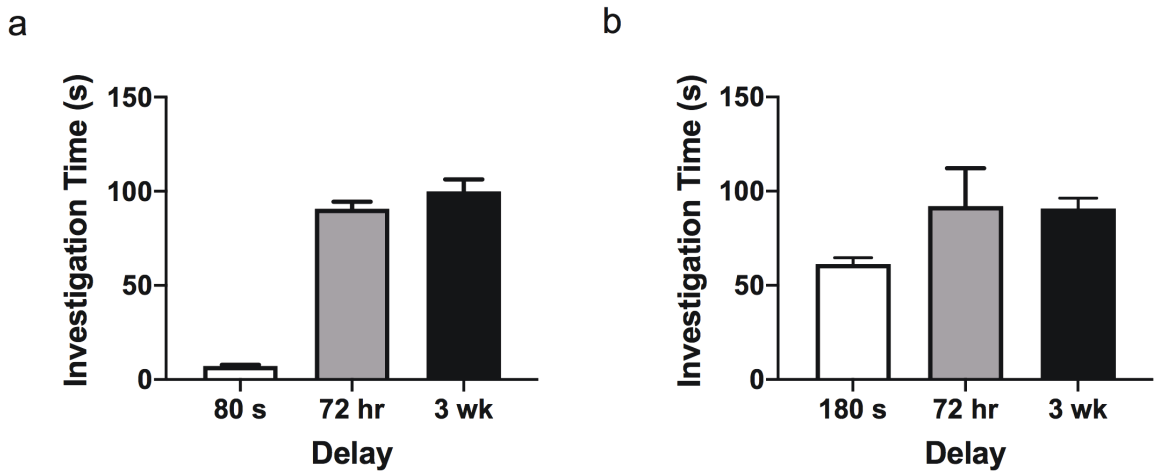

Figure 3.5. Mean (± SEM) time spent investigating objects during the familiarization phase for both the (a) mDNMS task and (b) NOP test.

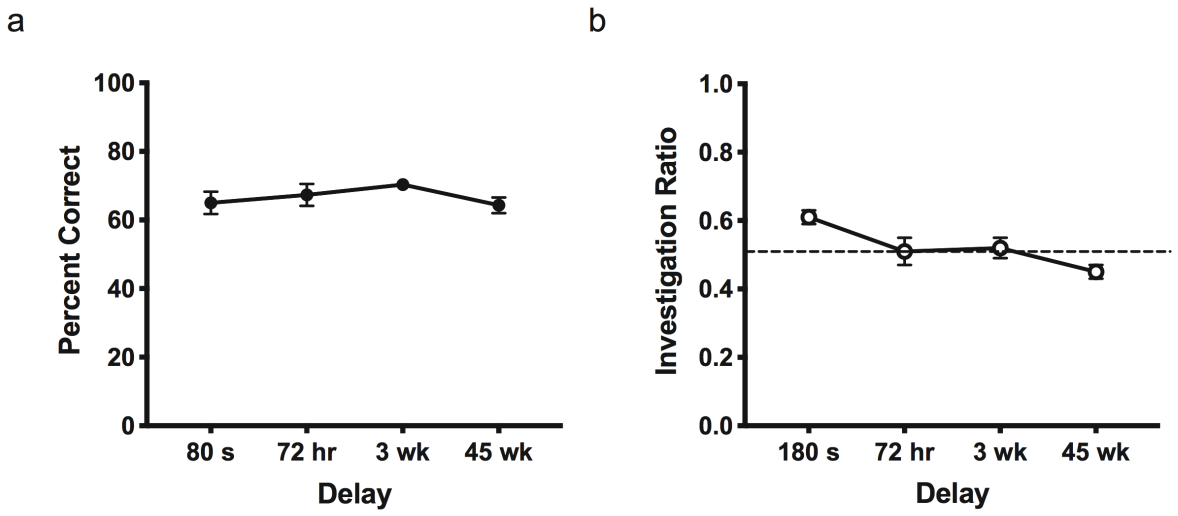

Figure 3.6. Average scores (± SEM) on the (a) mDNMS task and (b) NOP test across four delays. Dashed line represents chance performance.

3.3.3. NOP mixed-delay testing

One 3-week delay trial was excluded for all rats because they showed an inherent preference for one object in the pair, and the last two trials of the ~45-week delay were excluded for all rats due to human error. Investigation ratios were calculated separately for each cumulative minute of the 5-min test. The investigation ratios for each delay are based on the first cumulative minute bin of the test whereby the group exhibited a statistically significant preference. Trials were excluded if a rat spent less than one second exploring either object on the test. This resulted in the exclusion of one trial for two rats during the 72-hr, and 3-week delay.

Figure 3.5b depicts the average time spent investigating objects during the familiarization phase for the 180-s, 72-hr, and 3-week delay. Rats spent on average 61.31 s (s = 8.83), 99.70 s (s = 48) and 90.73 s (s = 13.47) investigating objects during the familiarization phase for the 180-s, 72-hr and 3-week delay, respectively.

Figure 3.6b depicts the mean investigation ratios on the test at each delay. One-sample t-tests (one-tailed) performed on the first 3-min of the test revealed that rats had a mean investigation ratio significantly above chance during the 180-s delay ($t_{(6)}$ = 6.06, p = .001, Hedge's g = 3.31, 95% CI [1.62, 5.0]). Conversely, rats did not have investigation ratios significantly above chance level of performance on either the 72-hr ($t_{(6)}$ = .79, p > .05, Hedge's g =.44, 95% CI [-0.62, 1.5]) or 3-week delay ($t_{(6)}$ = .38, p > .05, Hedge's g =.16, 95% CI [-0.89, 1.21]). Lastly, based on the first minute of the test, rats had a mean investigation ratio significantly below chance during the ~45-week delay ($t_{(6)}$ = -2.03, p = .04, Hedge's g = -1.18, 95% CI [-2.23, -.03]), indicating a sample object preference on the test.

3.3.3.1. NOP test correlational analyses. Pearson correlations were computed to assess the relationship between total time spent investigating the sample objects during the familiarization phase and the average investigation ratios on the test for the 180-s, 72-hr, and 3-week delay NOP tests. There were no significant correlations (r = -.60 to -.06, p = .07 to .82), indicating that the magnitude of the novelty preference on the test was unrelated to the amount of sample investigation during the learning phase of the trial.

3.3.4. Correlation between scores on the mDNMS task and NOP test

Figure 3.7 shows the results of a correlational analysis of NOP investigation ratios and mDNMS scores. We compared average investigation ratios from the NOP trials with average

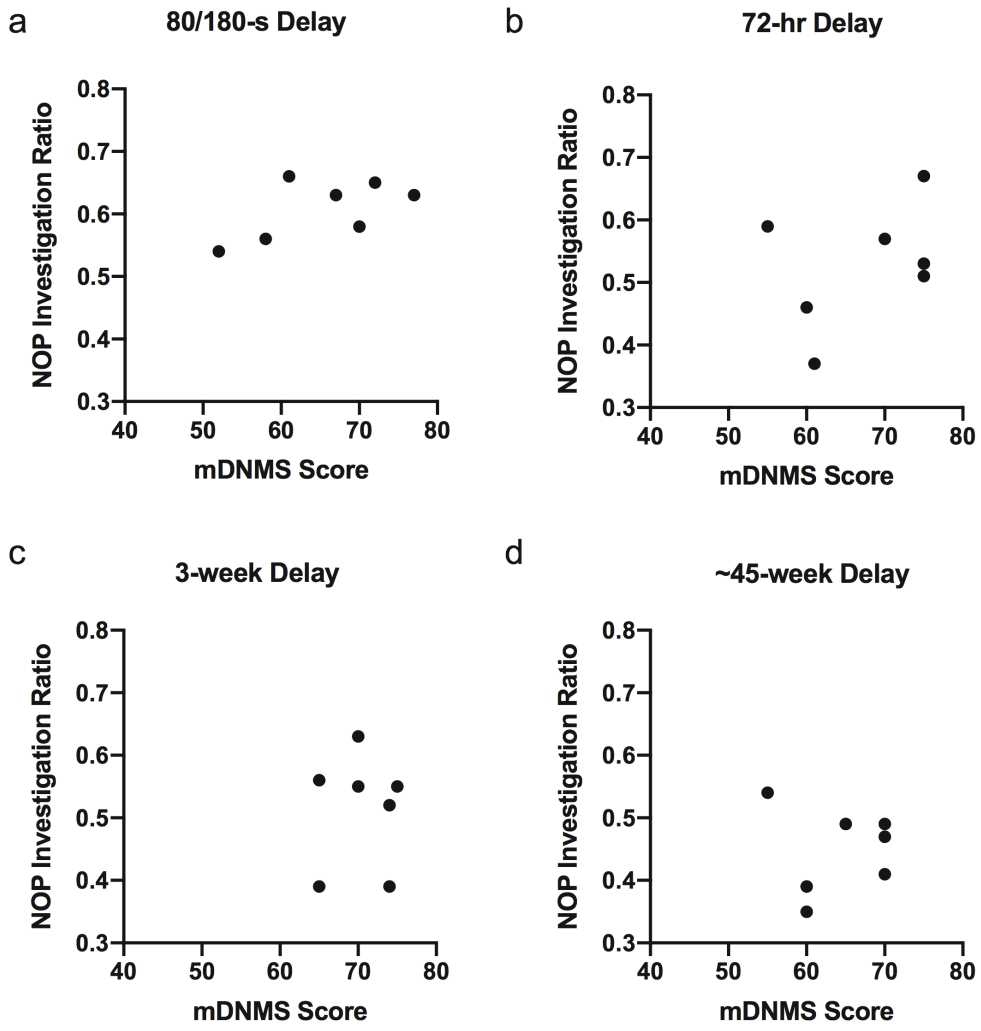

Figure 3.7. Scatterplots depicting the correlation between scores on the mDNMS task and NOP test following the (a) shortest, (b) 72-hr, (c) 3-week, and (d) ~45-week delay. Scores on the two tests were not significantly correlated on any of the delays.

percent-correct on the mDNMS task at each respective delay. There was no significant correlation between average NOP investigation ratios and average mDNMS scores on the short delay (r = .60, p = .08, 95% CI [-.28, .92]), 72-hr delay (r = .37, p = .21, 95% CI [-.53, .88]), 3-week delay (r = .05, p = .46, 95% CI [-.73, .77]), and ~45-week delay (r = -.003, p = .50, 95% CI [-.75, .75]).

3.4. Discussion

The accuracy scores on the mDNMS task were significantly above chance following the 72-hr, 3- and ~45-week delay, indicating that, on average, rats recognized the sample objects following all three retention intervals. The present results are to our knowledge, the first successful attempt to employ a reinforcement paradigm to assess object-recognition memory in rats following retention intervals lasting longer than several days. The fact that rats showed intact memory for objects encountered up to 45 weeks earlier is intriguing because it indicates that the mDNMS task can prove useful for examining questions regarding memory for objects learned at various time points over the course of one year. This is something that has not been feasible with the existing object-recognition tests. Thus, the practical significance for neurobiological studies of object recognition is that mechanisms of long-term memory that operate over periods of several days, weeks, and even up to a year can potentially be studied using the mDNMS task methods described in the present study.

On the NOP test, only the mean investigation ratios on the 180-s delay were statistically significantly above chance. The rats' failure to exhibit a novel-object preference on the test following both the 72-hr and 3-week delay tests was unexpected. The reason why rats failed to exhibit a novelty preference cannot be determined from the available data, thus it is unclear whether or not this reflects a failure to recognize the sample objects on the test. The above chance level accuracy scores on the mDNMS task, however, provide clear evidence that the rats did in fact recognize objects learned 72 hr and 3 weeks earlier. The divergent results between the mDNMS task and NOP test cannot be explained by differences in the familiarization procedure because it was identical for both tasks. Moreover, the results cannot be explained by differences in the amount of time the rats spent investigating the sample objects during the familiarization phase for both tasks in the circular-track because the rats spent a similar amount of time investigating the sample objects used on both tasks. Based on the mDNMS task results, we can affirm that this procedure of repeatedly exposing rats to the sample object over consecutive days

worked as a means to measure memory for objects over long delays. Collectively, the findings suggest that simply relying on a rat's natural tendency to investigate unfamiliar objects over familiar ones may fail to provide an accurate estimate of its object-recognition abilities. Accordingly, one should be cautious when concluding that a lack of novelty preference signifies a deficit in object-recognition memory.

The change in context between the familiarization and test phase on the NOP may explain why rats failed to exhibit a novelty preference on the test. On standard NOP tests using long retention intervals, the familiarization and test phase are administered in the same open field arena, and thus the same environmental context. Changing the context between learning and testing has been found to disrupt rats' novel-object preferences (Dellu, Fauchey, Le Moal, & Simon, 1997). Previous research has shown that performance on recognition tests improves when the test is conducted in the same context in which the original learning occurred compared to a different context (Baddeley, 1975). This context-dependent memory is hypothesized to occur because when stimuli are encoded, the context is encoded along with them, and a configural representation is formed in the brain. When the test subject is returned to the same context for a test, the familiar environment reactivates the configural representation, which somehow facilitates retrieval of the representation of target stimuli (Hirsch, 1974). Thus, perhaps the rats failed to recognize the sample object when it was encountered in a different environmental context. Others have argued, however, that a failure to retrieve information from memory following a change in context does not make sense when you consider animals live in forever changing environments (Devenport, 1989). Accordingly, an alternative explanation is that the change in context may introduce competing behaviours, which may change performance for reasons unrelated to a failure in memory. For example, the rat might spend more time exploring features of the apparatus or the sample object, consequently reducing investigation of the novel object. Accordingly, this may explain why the rats in the present experiment failed to preferentially explore the novel object on the 72-hr and 3-week delay tests. Although these results fail to confirm with certainty whether or not rats recognized the sample objects, they do reveal the problems interpreting the status of object-recognition memory by simply relying on a rats' tendency to explore familiar and unfamiliar objects.

The significant preference to investigate the *sample* object on the test following the ~45-week delay was unexpected. Based on these results we can conclude that the rats recognized the

sample objects because if they did not, then equal time should have been spent investigating both objects on the test, as both objects would have been equally unfamiliar. The objects on the ~45-week delay, however, were not counterbalanced and the same objects were used as samples for all rats. Thus, it is possible that the rats had an inherent preference for the sample objects on the NOP test. We are confident that this was not the case, because two of the three object pairs used in the present experiment had been previously screened for preference by a different group of rats in a nonchoice test, and we found no significant inherent preference for either one of the objects in the pairs. Moreover, based on our observations, no object in the remaining pair appeared to elicit a strong inherent preference during the test. Thus, the results provide additional support for the argument that the magnitude of a novelty preference should not be used to gauge the strength or persistence in memory for the sample object (Gaskin et al., 2010). Specifically, interpreting the magnitude of a novel-object preference on the test as indicative of the strength in memory for the sample object implies that a rat with a high novelty preference has superior memory for an object compared to a rat that exhibits a low novelty-preference. However, by this logic, the rat with a low novelty preference would be exhibiting a bias to explore the sample object, which can equally be taken to reflect object-recognition memory. Accordingly, assuming that the magnitude of the novelty preference is directly proportional to the strength in memory for the sample object is illogical because it fails to account for low novelty preferences (i.e., strong sample preference) being indicative of intact object-recognition memory.

The lack of a statistically significant correlation between scores on the mDNMS task and NOP test suggest that the two behavioural tasks do not tax the same underlying construct, one of which is object-recognition memory. The extent to which the behavioural tasks provide a measure of object-recognition abilities differs. On the mDNMS task the rat is presented with a sample object to learn, followed by a test whereby it makes a choice between displacing either the sample or a novel object. By training the rat to learn that it will receive a food reward when it displaces the unfamiliar object on the test, it provides the opportunity to instruct the rat on the purpose of the task. Accordingly, when a rat exhibits a tendency to displace unfamiliar objects over familiar objects on the test to a high criterion, then you can be more certain that it is engaging in this behaviour because it has learned the reward contingency and is applying it. Consequently, the observed behaviour on the test can more confidently be taken to reflect the rat's ability to discriminate between familiar and unfamiliar objects. Conversely, using a novelty

preference score as an index of a rat's object-recognition abilities does not provide to the same level of certainty that this behaviour reflects the accuracy of the rat's memory for the sample object. Intuitively, when comparing rats' performance across dozens of trials on the mDNMS task, and one rat obtains 90% correct choices and another rat gets 60% correct, it is easier to accept that the first rat has better recognition memory than the second rat. Conversely, on the NOP test when comparing a 90% novelty preference ratio in one rat to a 60% novelty preference ratio in another rat, it is not so easy to make the same assumption. Accordingly, it is difficult to confidently claim that the degree of novel-object preference that a rat exhibits provides a reflection of its strength in memory for the sample object. Conversely, a less ambiguous reflection of a rat's object-recognition memory is one whereby the rat has been trained to make an explicit choice response based on memory, and is rewarded for accurate choices, such as on the mDNMS task. Thus, the observed scores on the mDNMS task provide a more straightforward estimate of a rat's object-recognition abilities compared to scores on the NOP test. Accordingly, the lack of correlation between scores on both tasks, likely reflects issues regarding the internal validity of the NOP test as an estimate of rats' object-recognition abilities.

Rats performed significantly above chance on both the probe tests and normal tests, suggesting they were not relying on olfactory cues to successfully locate the food reward on the test. The scores on the normal tests and probes, however, declined relative to scores obtained on the criterion sessions during task acquisition. This disruption in performance may reflect the disruptive effects of probe tests. Administering ten consecutive probe test sessions in combination with regular tests likely disrupted performance on the test because the rats learned that the novel object was no longer consistently rewarded. Previous delayed matching-to-sample studies suggest that animals can anticipate trial features such as the quantity and probability of a reinforcer, especially when trial features remain constant over many trials (Honig & Dodd, 1986). Consequently, changes made to features of a trial can affect an animal's performance, such that task accuracy may decline despite intact recognition abilities. Thus, the decline in accuracy on the normal tests during probe testing may have reflected the rat's incentive to respond accurately, and not necessarily memory abilities or the ability to detect the odor of the reward. In the future, a better design may include baiting both the sample and novel objects with a reward on the test but only making the reward underneath the novel object accessible, thus eliminating the need to conduct probe tests.

Overall, the results from present experiment demonstrate that the mDNMS task is a promising tool to assess long-term object-recognition memory in rats. Moreover, the degree of *novelty* preference on the NOP test should not be uncritically taken to reflect the strength in memory for the sample object, as the expression of this behaviour may vary for reasons unrelated to a failure in object-recognition memory. Indeed, the problem with simply relying on a rat's preference to investigate a novel object over a familiar one is that it makes it difficult to rule out alternative explanations for when a rat fails to exhibit this preference on the test. Conversely, requiring a rat to perform many trials each of which involve making an explicit choice response between familiar and unfamiliar objects, and giving a reward for accurate choices, provides a less ambiguous interpretation of object-recognition memory. Indeed, there are considerably fewer alternative explanations, besides object-recognition memory, to explain the observed behaviour on the test.

Chapter 4: Effects of perirhinal cortex and hippocampal lesions on rats' performance on two object-recognition tasks

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Abstract

The effects of hippocampal (HPC) damage on rats' novel object preference (NOP) performance have been rather consistent, in that HPC lesions do not disrupt novelty preferences on the test. Conversely, there have been inconsistent findings regarding the effects of perirhinal cortex (PRh) lesions on rats' novel-object preferences. Given the concerns that have been raised regarding the internal validity of the NOP test, viz. that the *magnitude* of the novel-object preference does not necessarily reflect the strength in memory for an object, it could explain the discrepant findings (Gaskin et al., 2010; Gervais et al., 2013, 2016; Gulinello et al., 2018). The goal of the present experiment was to examine the effects of PRh and HPC lesions on rats' object-recognition memory using the new modified delayed nonmatching-to-sample (mDNMS) task, as it circumvents the interpretational problems associated with the NOP test. Rats received PRh, HPC, or Sham lesions and were trained on the mDNMS task using a short delay (~30 s). Both PRh and HPC rats acquired the task at the same rate as Sham rats, and reached a similar level of accuracy, indicating intact object-recognition. Thereafter, rats were tested on the NOP test using a 180-s delay. Rats with HPC lesions exhibited significant novel-object preferences, however, both the PRh and Sham rats failed to show a novelty preference. The discrepancy in both the PRh and Sham rats' performance on the mDNMS task and NOP test raises concerns regarding the internal validity of the NOP test, in that the magnitude of a rat's novel-object preference does not accurately reflect the persistence or accuracy of a rat's memory for the sample object.

4.1. Introduction

The hippocampus (HPC) and perirhinal cortex (PRh) are two medial temporal lobe (MTL) structures implicated in object-recognition memory. Early attempts to model human MTL amnesia in animals primarily focused on damaging the HPC because correlational findings from human amnesic patients suggested the extent of HPC damage was associated with the degree of memory impairment (Milner et al., 1968; Scoville & Milner, 1957). The first successful animal model of amnesia involved the use of the *delayed nonmatching-to-sample* (DNMS) task using trial unique stimuli. On a DNMS trial the animal is briefly presented with a sample object and receives a reward for displacing it from over a food well. After a retention interval, the sample is now presented alongside a novel object. This time, the animal receives a reward if it selects the novel object. Different sample and novel objects are used on each trial, so reliably accurate performance requires that the animal recognize the sample object. Early findings from nonhuman primate models supported the idea that the HPC played an important role in object-recognition memory because damage to it produced DNMS impairments (Gaffan, 1974; Helen Mahut, Zola-Morgan, & Moss, 1982; Mishkin, 1978; Murray & Mishkin, 1984; Zola-Morgan & Squire, 1985, 1986; Zola-Morgan, Squire, & Amaral, 1989a). However, other studies conducted around the same time revealed that HPC damage did not impair DNMS performance in either nonhuman primates (Murray & Mishkin, 1986; Zola-Morgan, Squire, Amaral, et al., 1989) or rats (see Mumby, 2001 for a review). For example, Aggleton, Hunt, and Rawlins (1986) showed that rats with HPC lesions successfully acquired the DNMS task at the same rate as control rats and reached a similar mean accuracy score on the task following the maximum delay of 60 s. Comparatively, Mumby, Wood, and Pinel (1992) found that rats with either separate or combined lesions of the HPC and amygdala performed comparable to controls on the DNMS task at delays lasting up to 120 s, and only showed a slight decline in performance following a 600-s delay. Over time, it was revealed that the observed DNMS impairments in nonhuman primates was likely the result of incidental damage made to the rhinal cortices (PRh and entorhinal cortex), as severe DNMS deficits were observed in nonhuman primates following damage made to either the PRh (Meunier, Bachevalier, Mishkin, & Murray, 1993; Zola-Morgan, Squire, Clower, & Rempel, 1993) or rhinal cortices (Gaffan & Murray, 1992; Meunier, Bachevalier, Mishkin, & Murray, 1993; Mishkin & Murray, 1994; Murray & Mishkin, 1986; Suzuki, Zola-Morgan, Squire, & Amaral, 1993; Zola-Morgan et al., 1989).

Only a few studies have been published on the effects of either rhinal cortex or PRh damage on rats' DNMS performance (Barnes, Floresco, Kornecook, & Pinel, 2000; Mumby & Pinel, 1994; Wiig & Bilkey, 1995), but they are consistent with findings from nonhuman primates. For example, Mumby and Pinel (1994) first trained rats on the DNMS using delays ranging from 4 to 600 s. Afterwards, rats received rhinal cortex lesions and were retested. The rats' post-surgery scores on the 4-s delay were comparable to their pre-surgery scores, however, their scores on delays lasting 15 s or longer had significantly declined. Using a similar DNMS paradigm, Wiig and Bilkey (1995) administered lesions restricted to the PRh *prior* to training rats on the DNMS task. Compared to sham-operated rats, PRh lesion rats required more trials on average to reach the performance criterion (362 vs. 174), but this difference was not statistically significant. When the rats were tested using different delays ranging from 4 to 120 s, rats with PRh lesions had scores that were significantly lower than the Sham rats following delays lasting 30 s or longer (Wiig & Bilkey, 1995). Accordingly, the findings from DNMS tasks suggested that an intact rhinal cortex or specifically PRh, is important for normal object-recognition memory.

Beginning in the early 1990s, researchers began using the *novel object preference* (NOP) test to examine the effects of discete lesions made to either the HPC or PRh on object-recognition. Before describing the findings, it is important to first understand the different ways that NOP results are interpreted. On the NOP test, a novelty-preference score (e.g., investigation ratio) is calculated based on the amount of time the rat spends investigating the novel object compared to the sample object. When two or more groups of rats are tested (i.e., a treatment and control group), the investigation ratio can be statistically analyzed using two different methods: a "within-subjects" analysis to compare a group's average score to what would be expected by chance level of performance or a "between-subjects" analysis to compare the average score of each group to one another. Using the former method, object-recognition memory is inferred when a group score is statistically significantly above chance. This indicates that on average, the group spent more time investigating the novel object compared to the sample, indicating they recognized the sample object. By comparison, using the between-subjects method, when there is a statistically significant difference between groups, the group with the lower mean score is presumed to have an object-recognition impairment. Furthermore, this inference is made regardless of whether or not the lower mean score is significantly above chance.

When inferring the status of memory based on the relative degree of novel-object preference, it suggests that high preference scores are indicative of superior memory for the sample object. This interpretation, however, is problematic because to date there is no evidence to suggest that the degree of novel-object preference reflects the strength in memory for the sample object (Gaskin et al., 2010; Gervais et al., 2013, 2016; Gulinello et al., 2018). For example, the amount of time rats spend investigating the sample object during the familiarization phase does not predict the degree of their novel-object preference. Moreover, providing rats with extended exposure to a sample object does not influence their degree of novelty preference (Gaskin et al., 2010; Gervais et al., 2013, 2016). Both of these findings are counter to two major assumptions that underlie the way in which NOP data are typically interpreted: 1) that rats are encoding the sample object features when investigating it during the familiarization phase, and 2) that the magnitude of the novelty preference reflects the strength in memory for the sample object. Accordingly, interpreting differences in the magnitude of novelty preference scores as a reflection of differences in object-recognition abilities should be avoided. Although researchers using the between-subjects analysis typically include a within-subjects test in the results section, oftentimes the interpretation of results is based solely on the between-subjects analysis. Evidently, interpreting the effects of some treatment can yield different conclusions depending on the type of comparison used (within vs. between).

The effects of HPC damage on rats' object-recognition memory using the NOP test have been fairly consistent. Similar to control rats, rats with HPC lesions still exhibit significant novel-object-preferences on tests following delays lasting several minutes to 24 hr (see Cohen & Stackman, 2015 for a review; Ennaceur & Aggleton, 1994; Ennaceur et al., 1996; Ennaceur & Aggleton, 1997; Forwood, Winters, & Bussey, 2005; Gaskin, Tremblay, & Mumby, 2003; Mumby, Gaskin, Glenn, Schramek, & Lehmann, 2002; Winters et al., 2004). One group of researchers reported that HPC lesions produced NOP deficits following delays lasting longer than 10-min, which the authors interpreted as an object-recognition impairment (Clark et al., 2000). In this case, the HPC group had average scores that were significantly lower relative to the control group, however, their scores were statistically significantly above chance, clearly indicating that they successfully discriminated between novel and sample objects on the test, even after a 24-hr delay. Despite this report, most researchers that have conducted experiments aimed at testing the hypothesis that HPC damage impairs object-recognition memory have failed

to find support for it (see Mumby, 2001 for a review). Overall, interpreting the effects of HPC lesions on object-recognition memory using the standard NOP test has been rather straightforward because HPC rats maintain significant novelty preferences, which clearly indicates intact object-recogniton memory. These findings taken together with those from DNMS studies suggest that the HPC does not play a critical role in object-recognition memory. There is evidence, however, to suggest that an intact HPC is critical for the ability to remember the specific location of familiar objects within a particular context (O'Brien, Lehmann, Lecluse, & Mumby, 2006; Piterkin et al., 2008).

The majority of behavioural experiments assessing the effects of PRh damage on rats' object-recognition memory have relied on using the NOP test, not the DNMS task. Determining the effects of PRh lesions on rats' object-recognition memory using the NOP test has not been straightforward, and this stems from problems interpreting behaviour on the test. PRh lesions have been reported to produce object-recognition impairments because rats with PRh lesions exhibit significantly lower novel-object preferences compared to control rats, despite the scores being significantly above chance (cf. Bussey, Duck, Muir, & Aggleton, 2000; Bussey, Muir, & Aggleton, 1999; Clark, Zola, & Squire, 2000; Norman & Eacott, 2004; Winters, Forwood, Cowell, Saksida, & Bussey, 2004). However, interpreting these results using the within-subjects comparison would lead one to conclude that PRh lesions failed to disrupt object-recognition memory given the observed bias to investigate the novel object. Accordingly, depending on the method used to interpret NOP data, the conclusions drawn can be rather different.

Nonetheless, upon closer inspection of the within-subjects results from several studies, there are discrepancies in terms of whether or not PRh lesions consistently abolish novel-object preferences. For example, some studies have shown that rats with PRh lesions, or combined postrhinal lesions, fail to exhibit statistically significant novelty preferences on the test (Aggleton, Keen, Warburton, & Bussey, 1997; Barker & Warburton, 2011; Ennaceur, Neave, & Aggleton, 1996; Ennaceur & Aggleton, 1997; Liu & Bilkey, 2001). Ennaceur and colleagues (1996) found that rats with PRh lesions exhibited a statistically significant novelty preference following a 1-min delay but not a 15-min delay. This finding was later replicated using a different group of rats (Ennaceur & Aggleton, 1997). Conversely, other studies revealed that rats with PRh lesions can exhibit significant novelty preferences following delays lasting up to and including 15 min (Bussey, Duck, Muir, & Aggleton, 2000; Bussey et al., 1999; Ennaceur et al.,

1996; Ennaceur & Aggleton, 1997; Norman & Eacott, 2004, 2005). In fact, depending on the type of object or the test minute bin that is analyzed, rats with PRh lesions can exhibit significant novel-object preferences following delays lasting one hour (Norman & Eacott, 2004), two hours (Barker, Bird, Alexander, & Warburton, 2007) and even up to 24 hours (Winters et al., 2004). These findings reveal that rats with PRh damage do in fact successfully discriminate between novel and sample objects on the test, indicating intact object-recognition.

The discrepant findings cannot be explained by differences in lesion size nor methodological procedures, as they are similar across experiments (some of the inconsistent results even occurred within the same study). The discrepancies likely stem from the interpretational problems associated with any test of incidental learning in which an animal incidentally explores. Indeed, a lack of bias to explore a novel object on the test following some form of treatment can reflect an object-recognition impairment, but it can also reflect some other factor. For example, a treatment may alter the behavioural expression of this novelty preference for reasons unrelated to failures in object-recognition memory. For example, a treatment may affect a rat's stress response, which can alter the extent of spontaneous investigation of a novel object. Indeed, higher stress levels reduce a rat's tendency to approach novel objects (Gulinello et al., 2018; Hughes, 1997). Alternatively, the tendency to investigate novel objects may decline due to an increase in competing behaviours. For example, a treatment may disrupt a rat's spatial memory while maintaining intact object-recognition memory. Rats' tendency to investigate novel objects changes a function of environment familiarity (Besheer & Bevins, 2000; Sheldon, 1969). Consequently, novel object investigation may decline due to an increase in exploring features of the apparatus or the sample object. Determining potential reasons for why a rat fails to display a novelty preference on the test is confounded by the fact that the NOP test does not involve a goal, and thus the rat is not required to make an explicit choice response based on memory. Indeed, the lack of an instrumental response complicates the interpretation of the behaviour because it fails to limit the number of alternative explanations besides an object-recognition memory impairment for the observed behaviour on the test. Overall, the effects of PRh lesions on rats' novel-object preference have been rather inconsistent, making it difficult to confirm whether or not PRh lesions disrupt object-recognition memory in rats.

Considering both the HPC and PRh have been implicated in object-recognition memory, it was important that we determine the effects of HPC and PRh lesions on rats' performance on

the new modified delayed nonmatching-to-sample (mDNMS) task. Additionally, given the inconsistent findings on the effects of PRh lesions using the NOP test, we also decided to administer NOP tests and to compare their performance to that on the mDNMS task. Rats received either PRh or HPC lesions and were trained on the mDNMS task using a short retention interval (~30 s). Afterwards, the rats were tested on a standard NOP test using a 180-s retention interval. Based on previous findings, we predicted HPC rats would perform similarly to control rats on the mDNMS task and would exhibit significant novelty preference on the NOP test. In terms of the effects of PRh lesions on mDNMS performance, we predicted PRh rats would require significantly more trials to reach the performance criterion and would have significantly lower scores compared to control rats. Given the inconsistent findings on the effects of PRh lesions on rats' novel-object-preference, we were unsure as to whether or not PRh lesions would disrupt novel-object preference on the NOP test.

4.2. Materials and Method

4.2.1. Subjects

The subjects were 14 male Long-Evans rats (Charles River, St. Constant, QC), weighing 500-575 g (~28 weeks old) at the start of the experiment. The rats were housed in polypropylene cages (48 × 25 × 20 cm) in a colony room under a reverse 12:12 light-dark cycle, with light onset at 8:00 p.m. The rats had continuous access to water and each received a daily ration of ~25 g of rat chow (Charles River Rodent Animal Diet, no. 5075) in the late afternoon, after behavioral testing was finished for the day. Prior to surgery, rats were pair-housed, and following surgery they were individually housed. Prior to this experiment, nine of the rats were briefly used in a separate study, which involved administering a brief puff of air to the face after approaching a stimulus object. The object was not used in the present experiment, and each of the nine rats was distributed equally into each group. All procedures were approved by the Concordia University Animal Care and Use Committee, and were in accordance with the guidelines of the Canadian Council on Animal Care.

4.2.2. Surgery

Hippocampal surgical lesions (HPC group, $n = 5$) and perirhinal cortex lesions (PRh group, $n = 5$) were performed while the rats were anesthetized with isoflurane gas (0.8 l/min oxygen at 14.7 psia at 21º, Janssen, Toronto, ON). The rats were secured in a stereotaxic frame and a midline scalp incision was made to expose the skull. The lesions were made by injecting

N-methyl-D-aspartic acid (NMDA; Sigma Chem. Co., St. Louis, MO) at 5 sites bilaterally for the HPC (AP -3.1, -4.1, -5.0, -5.3, -6.0; ML ±1.5, ±2.8, ±3.0, ±5.2, ±5.0; DV -3.6, -4.0, -4.5, -7.0, -7.3), and 4 sites bilaterally for the PRh (AP -3.3, -4.3, -5.3, -6.8; ML ± 4.8; DV -9.35, -9.35, -9.35, -9.2) based on (Paxinos & Watson, 1998). A 26-gauge injection cannula connected to PE-20 tubing was attached to a 10 µl Hamilton syringe mounted on a micro-injection pump (KD Scientific). The HPC lesions were made by infusing NMDA (5.1 mM solution, dissolved in 0.1 M phosphate buffered saline, final pH = 7.3) at a flow rate of 0.15 µl/min until a total volume of 0.4 µl was reached at each site. The PRh lesions were made by infusing 0.25 µl NMDA at each injection site using a flow rate of 0.1µl/min, and the infusion cannulas were lowered from the dorsal surface using a 20° angle to the vertical plane for the first three sites and a 22° angle for the final site. For both lesion types, the injection cannula remained in place for an additional 2.5 minutes before being slowly retracted. Following the surgery, the incision was closed using wound clips and a topical antibiotic (Hibitane, Wyeth Animal Health, Guelph, ON) was applied to the incision area. Each rat in the HPC group received an injection of diazepam (10mg/kg, IP; Hoffmann-La Roche, Mississauga, ON) as a prophylaxis against seizures. The control rats (Sham group, $n = 4$) received similar treatment, except no damage was made to the skull or brain. All rats received Penicillin G Procaine (0.2 ml, SC; Vétoquinol N.–A Inc., Lavaltrie, QC) and Ketoprofen (5 mg/kg, sc; Merial Canada, Baie d'Urfé, QC) post-surgery. Rats were given a 2-week recovery period prior to continuing behavioural testing. Rats were provided ad lib food during the first 10 days following surgery. For the remaining four days, rats were fed 25g of food at their usual mealtime.

4.2.3. Apparatuses

4.2.3.1. mDNMS task. A large multi-level environment (152 × 145 × 86 cm) was used to test the rats (Figure 4.1). The apparatus was a modified, freestanding steel cage rack, enclosed on three sides by wire mesh, with a removable, clear acrylic front panel. The apparatus had five levels, each covered with woodchip. The top four levels were divided into two equal halves by a plastic barrier wall, and the bottom level remained undivided. A loading cage (58 × 37 × 20 cm) was placed on the top left side of the apparatus. A rat entered the apparatus via a hole in the bottom of the loading cage that was placed over a passageway leading to the top level of the

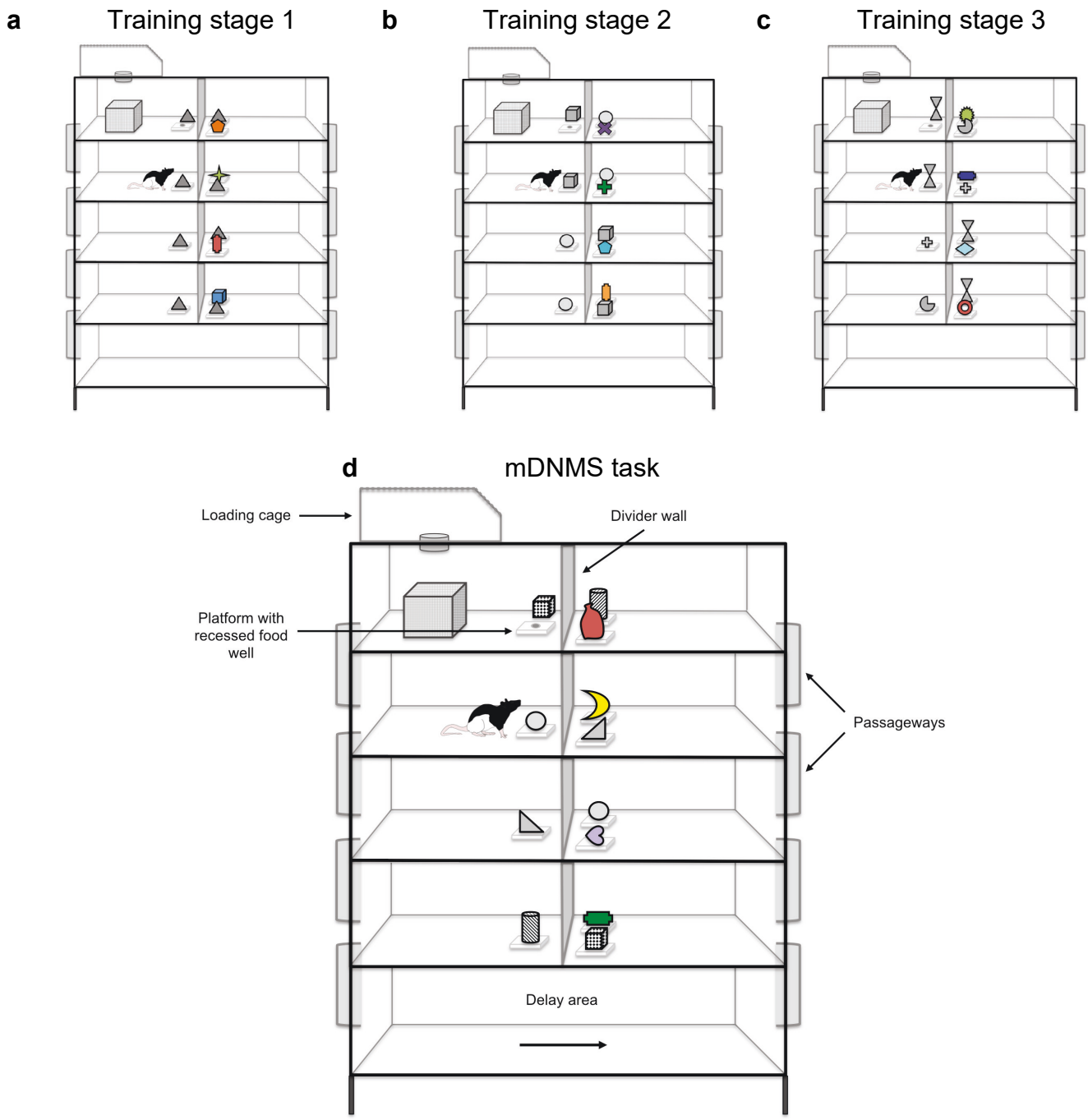

Figure 4.1. Diagram of the apparatus used for mDNMS testing depicting a session on (a-c) training stage 1, 2, and 3, respectively, and (d) mDNMS task acquisition. A loading cage provided access to the apparatus, and passageways on both sides of the apparatus allowed rats to access the different levels. The top four levels contained plastic platforms with recessed food wells in which objects could be placed over. During training, the rat descended the left side of the apparatus encountering either (a) four copies of one sample object on stage 1, (b) two copies of two distinct sample objects on stage 2, or (c) two copies of one sample object and two distinct sample objects on stage 3. During (d) mDNMS task acquisition, the rat encountered four unique sample objects as it descended the left side. For all stages (a-d) once the rat reached the bottom level, it traversed to the right side where it ascended each level encountering 4 different tests. On each test a copy of the sample object was paired with a unique novel object.

apparatus. Rats traversed the different levels via wire mesh passageways located on both sides of the apparatus. The design of the apparatus was such that a rat had to climb down the passageways on the left side of the apparatus in order to gain access to the right side, which it then could ascend from level to level. The top four divided levels contained plastic rectangular platforms (30 × 12 × 1 cm) each with a recessed food well (2 cm in depth), over which stimulus objects could be placed. One platform was placed on each level on the left side of the apparatus, and on the right side of the apparatus, two platforms were placed on each level with the food wells 9 cm apart. All platforms were positioned near the middle barrier wall, in line with the passageway that provided access to the level. The room contained dim lights (40 lx) and one video camera was positioned in front of the apparatus to record test sessions.

4.2.3.2. NOP test. The apparatus for the NOP test was an open-field arena (60 × 70 × 70 cm), constructed of grey PVC plastic. The floor of the arena consisted of a stainless-steel tray covered with woodchips. The floor was removable via a slot at the bottom of one wall to facilitate changing the woodchips between each trial. The testing room contained dim lights (14 lx) and a videocamera was positioned over the arena to record the sessions for later analysis.

4.2.3.3. Stimulus objects. A total of 320 different objects were used as stimuli for the mDNMS task. Objects were made of plastic, metal, glass, or glazed ceramic, and ranged in size from 4 to 18 cm in height, and 4 to 13 cm in width. Each object was large enough to cover the food well but light enough to be easily displaced by a rat. There were two copies of each mDNMS task object –one for the learning phase and one for the test. The objects were cleaned after every trial on which they were used, by wiping with a damp paper towel. At the end of each day the mDNMS task objects were cleaned using a diluted bleach solution (1:20 concentration ratio).

A separate pool of 6 objects was used for the NOP test. Objects for the NOP test were made of plastic, metal, glass, or glazed ceramic, and ranged in size from 7 to 18 cm in height, and 5 to 13 cm in width. There were at least three copies of each object–two for the familiarization phase and one for the test. A small glass jar (6 cm high) was attached to the bottom of each object with epoxy. The objects were fixed in place by screwing the jars into inverted lids that were attached to the stainless-steel tray in the open field arena (positioned 27 cm from opposing corners). The objects were cleaned after every trial on which they were used,

by wiping with a damp paper towel. At the end of each day the NOP objects were cleaned using a 70% ethanol solution.

4.2.4. Behavioural procedures

4.2.4.1. mDNMS task.

4.2.4.1.1. Pre-surgery habituation. The rats were handled for ~10 minutes daily for one week before they were habituated to the apparatus. Rats received one 30-min habituation session five days per week. All rats were placed in the apparatus with no stimulus objects present, and ~20 Cheerios (1.8 g, General Mills) were placed on each level near and inside the food wells. Once each rat was consistently eating Cheerios from each food well and reliably completing the entire circuit in less than ten minutes they moved onto the pre-surgery training. Rats required an average of 8 habituation sessions (min. = 6 and max. = 12).

4.2.4.1.2. Pre-surgery training. The rats were now tested individually and they were introduced to stimulus objects. The goal of this stage was to train rats to displace objects from over food wells and to dig for a buried Cheerio. A rat received one session per day that consisted of two phases: a sample phase and a choice phase. On the sample phase, the rat descended the left side of the apparatus and encountered four copies of one sample object. One Cheerio was placed in each food well to encourage the rat to approach and investigate the sample objects. On the choice phase, the rat ascended each level on the right side of the apparatus encountering a different novel object paired with a copy of the sample object and one Cheerio was placed in the food well under each novel object. On the first few sessions, the objects only partially covered the food well to encourage timid rats to displace objects. As sessions continued, the objects were gradually positioned to cover the entire food well. Once the objects covered the food wells, the Cheerios on the sample and choice phase were gradually buried beneath woodchip until the food well was entirely filled to the top (2 cm deep). On this stage, rats were not required to reach a performance criterion, rather they were simply trained until they consistently displaced objects and dug for buried Cheerios on two consecutive sessions, after which they received surgery. Rats required on average 20.29 (s = 3.31) training sessions at this stage.

4.2.4.1.3. Post-surgery training. Following recovery from surgery, the rats received the standard three stages of pre-training: stage 1, 2, and 3 (referred to as ‘training’ in the present experiment). Training stage 1, 2, and 3 differed in the number of distinct sample objects that were presented to the rat: one, two or three, respectively (see Figure 4.1a-c). Throughout the

behavioural testing, the experimenters testing the rats were blind to the surgical treatment for each rat. During training and subsequent stages, the experimenter left the room after placing the rats in the apparatus and watched the session on a TV monitor in an adjacent room.

At each stage, a rat received one session per day, which consisted of two phases: a sample phase and a test phase. On the sample phase, the rat descended the left side of the apparatus and encountered either four copies of one sample object (stage 1, which was identical to pre-surgery training), two copies of two different sample objects (stage 2), or three different sample objects –two copies of one object on the top two levels, and two distinct sample objects on the bottom two levels (stage 3). The objects completely covered the food wells, and one Cheerio was buried in woodchip in each food well. On the test phase, the rat ascended each level on the right side of the apparatus encountering a different novel object paired with a copy of the sample object and one Cheerio was buried in woodchip under each novel object. Thus, the test phase consisted of four separate 'trials', one for each level. On stage 2 and 3 the sample objects on the test phase were presented in the same order that the rat had encountered them on the sample phase (i.e., the first sample object appeared on the first test level). The sample and test phase were separated by a short retention interval in which the rat spent traversing the bottom level of the apparatus. On training stage 1 the mean delay (in seconds) for the Sham, HPC, and PRh group was 70 (s = 17.47), 61 (s = 49.4), and 34 (s = 12.76), respectively. On training stage 2 the average delay for the Sham, HPC, and PRh group was 33 (s = 16.92), 23 (s = 13.21), and 28 (s = 20.08), respectively. Lastly, on training stage 3 the average delay for the Sham, HPC, and PRh group was 28 (s = 23.27), 28 (s = 22.74), and 22 (s = 7.20), respectively. On all stages, a rat had to reach a performance criterion of 80% of trials correct on five consecutive sessions (16 trials correct out of 20) before advancing to the next stage.

A correct choice on a test trial was defined as the rat either displacing the novel object before displacing the sample object, or only displacing the novel object. An incorrect choice was defined as the rat only displacing the sample object, or displacing the sample object before the novel object. If a rat did not displace either object on a particular test, it was considered a non-trial. Different sample and novel objects were used on each session. On stage 1 a total of 15 different object sets were used, each containing 8 copies of one sample object and 4 unique novel objects. After 15 sessions, rats re-encountered the objects again in the same sequence, starting with the first object set. On stage 2 and 3 four new object sets were introduced –each containing

four copies of two distinct sample objects and four unique novel objects. These objects sets were used in combination with the stage 1 sets. The location of the novel object on the test phase was counterbalanced in a pseudorandom order.

After the rat completed the final test, the experimenter entered the room and removed the rat from the top right side of the apparatus. Between each rat, the woodchip on every level was redistributed to spread any potential odor cues left by a previous rat, and each object and plastic platform was cleaned using a 70% ethanol solution. A rat advanced to the next training stage once it reached a performance criterion of at least 80% of trials correct on five consecutive sessions (i.e., at least 16 correct trials out of 20 trials), or until it received a maximum of 50 sessions.

4.2.4.1.4. mDNMS task acquisition. During the final training stage, rats encountered four distinct sample objects, one on each of the divided levels of the sample phase (see Figure 4.1d). Thus, this stage was similar to conventional DNMS tasks in that each sample object was encountered only once during the sample phase and was subsequently paired with a unique novel object for the test phase. Similar to training, a session consisted of a sample and test phase. On the sample phase, a rat descended the apparatus to familiarize itself with four distinct sample objects, encountering a different one on each level. One Cheerio was buried in the food well under each sample object. During the test phase, a copy of each sample object was presented next to a novel object. A Cheerio was buried under the novel object on each test level. Each session consisted of four trials (as there were four distinct sample objects in the apparatus).

From this point forward a new collection of object sets was used. The objects changed on each session, however, the same objects served as the sample objects and novel objects for all rats. Once a particular object was used on a session, it was not used again until all of the objects in each set were used. This resulted in a particular object re-occurring approximately every 24 sessions. Moreover, an object that served as a sample the first time a rat encountered it, served as a novel the next time it was encountered (and vice versa). The sample and novel object on each trial were paired based on similarities in size, weight, and material. The location of the novel object on each test was counterbalanced in a pseudorandom order. A rat was required to reach a performance criterion of at least 80% of trials correct on five consecutive sessions (16 trials correct out of 20). The average delay between the sample and test phase for the Sham, HPC, and PRh group was 28 (s = 8.09), 26 (s = 17.62), and 21 (s = 8.35), respectively. On the final

criterion sessions, the average delay for the Sham, HPC, and PRh group was 34 (*s* = 19.69), 23 (*s* = 14.91), and 26 (*s* = 16.39), respectively. A rat was given a maximum of 60 sessions at this stage to reach the performance criterion. Rats received one session per day and were tested no fewer than five days per week. The dependent measures were mean percent correct choices and mean number of sessions required to reach the performance criterion.

4.2.4.1.5. Probe tests. Following testing, probe tests were administered to confirm the rats were not relying on olfactory cues to correctly locate the food reward buried under the novel object on the test phase. Two types of probe tests were conducted: 1) the food reward was omitted on the test (No Reward) and 2) the sample object was baited on the test (Sample-Baited). Two sessions (eight trials) of each type of probe test were performed and compared to two normal test sessions conducted contemporaneously. Probe tests were conducted over a 2-week period.

4.2.4.2. NOP test. After probe tests, the rats received NOP testing. Prior to NOP testing, rats were habituated to the open field arena for ten minutes daily for two consecutive days. Two identical objects were present in the open field arena during habituation. These objects were not used on subsequent experimental trials. Twenty-four hours following the last habituation session, rats received their first trial. A trial consisted of a familiarization phase and a test phase. For the familiarization phase, a rat was placed in the open field arena and allowed to explore two identical sample objects for five minutes. Following a 180-s retention interval, the rat was returned to the arena which then contained a copy of the sample object and a novel object, and the rat was allowed to investigate for five minutes. Objects were counterbalanced between rats such that the sample objects for approximately half of the rats were used as the novel objects for the remaining rats. The side in which the novel object appeared on was counterbalanced between rats and across trials for an individual rat. Each rat received three trials with a 180-s delay and trials were conducted on different days during a two-week period. Different object pairs were used for each trial, but the same object pair was used for all rats on corresponding trials. All three object pairs used in this experiment had been previously screened for preference by a different group of rats in a nonchoice test.

The rats were considered to be investigating an object if their head was 4 cm away from the object and oriented towards the object, or away from the object at no more than a 45º angle. A rat standing on its hind legs and touching the object with at least one forepaw was also

considered to be investigating. Climbing or sitting on top of an object was not considered investigation. The main dependent measure was the investigation ratio. This ratio compares the total object investigation time to the time spent with the novel object during the test phase (Ratio = [$Time_{novel}$/ ($Time_{novel}$ + $Time_{sample}$)]. To determine whether rats' discriminated between the objects, a one-sample *t*-test ($p < .05$) was used to compare mean investigation ratios to chance level of investigation (i.e., a ratio of 0.50). A ratio that was significantly greater than 0.50 indicated the rat spent more time investigating the novel object.

4.2.5. Histological Procedures

After behavioral testing was complete, the rats received a lethal dose of sodium pentobarbital (100 mg/kg, ip). They were transcardially perfused with 0.9% saline solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were excised and stored in a 4% paraformaldehyde/30% sucrose solution for at least 48 h until sectioning. The brains were sectioned at 40 μm through the HPC formation and PRh cortex using a cryostat microtome and every sixth section was mounted on glass microscope slides and stained using cresyl violet. A Cavalieri principle method was used to estimate the percentage of hippocampal and perirhinal cortex tissue that remained (Schmitz & Hof, 2005). Images of brain sections were taken using a Leica DMR-HC microscope mounted with a Hitachi 3CCD camera (model # HV-C20). Images were captured at 1.25× magnification and analyzed using ImageJ software (NIH). A total of 5 sections per rat (every third brain section) were sampled. The brain sections for the HPC group corresponded approximately to the following planes relative to bregma: -2.3, -3.3, -4.3, -5.3, and -6.3 mm, and for the PRh group they corresponded approximately to the following planes relative to bregma: -3.3, -4.3, -5.3, -6.0, and -6.8 mm. A point grid with an area per point of 0.2 mm^2 for the HPC counts, and a 0.1 mm^2 for the PRh counts, was randomly placed over each respective image and the total number of points in contact with HPC and PRh tissue was counted separately. To calculate the total estimated volume of spared HPC and PRh tissue in each rat, the sum of the number of points per section was multiplied by the area associated with each point, the section thickness, and the section sampling fraction. This value was turned into a percentage by dividing the estimated spared tissue volume by the average HPC or PRh volume of the Sham group, then multiplying by 100.

4.2.6. Statistical Analyses

Statistical analyses were performed using the *Statistical Program for the Social Sciences* (SPSS) software for Mac (IBM, version 22). The critical threshold for statistically significant results was set at $p < .05$. Eta-squared and Hedge's *g* are reported as measures of effect size. The 95% confidence intervals (CI) reported are calculated for the respective effect sizes.

4.3. Results

4.3.1. Data Screening

Before conducting any analyses, the data were screened according to the recommended best practices outlined by Kline (2009), and the statistical assumptions for each type of analysis were verified. All scores were standardized in order to detect the presence of outliers. A z-score greater than 3 was used to describe an outlier (Kline, 2009). Standardized scores for each variable did not reveal the presence of any outliers.

The normality of the distribution was assessed for each variable by measuring skewness and kurtosis. Scores were considered normally distributed with a skew less than 3 and a kurtosis less than 10 (Kline, 2009).The distribution of scores was also graphically assessed for normality using a histogram with a normal curve fitted to it. In the current sample, all variables showed acceptable skew and kurtosis, therefore no transformations were applied.

4.3.2. Histology and lesion quantification

Figure 4.2 depicts the largest and smallest HPC lesion (Figure 4.2a) and PRh lesion (Figure 4.2b). The NMDA infusions produced extensive cell loss in all principle subfields of the HPC and dentate gyrus (DG). The lesions were estimated to have removed 66.71% of the HPC (SEM = 2.56%; range: 60.08–73.00%). Damage to the dorsal HPC, including the DG, was complete in all but two cases. For those two rats, there was approximately 83% damage made to the dorsal HPC. There was also extensive damage to the ventral HPC, but this was more variable between hemispheres and animals. There was sparing of cells in the most posterior part of the subiculum in all rats. The injection cannulae caused minor damage to the posterior parietal cortex.

The PRh boundaries were defined according to (Burwell, 2001). The NMDA produced almost complete bilateral lesions in each PRh rat. The lesions were estimated to have removed 78.47% of the PRh (SEM = 2.40%; range: 70.28–84.90%). All PRh rats sustained minor bilateral damage to the anterior portion of the lateral entorhinal cortex. There was moderate bilateral

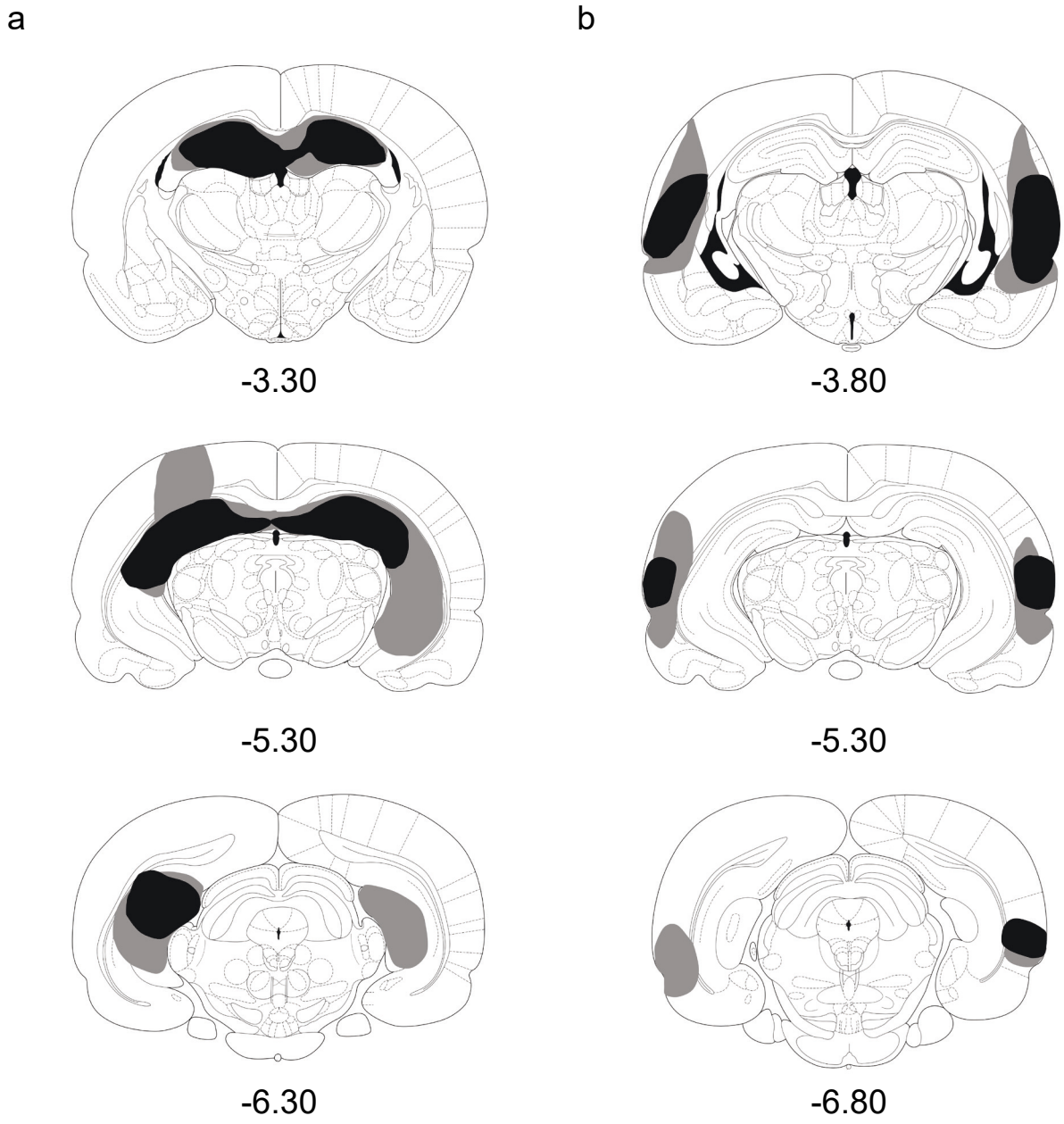

Figure 4.2. Coronal sections at three planes relative to bregma (in millimeters) depicting the extent of the smallest (black) and largest (gray) (a) HPC lesion and (b) PRh lesion. Drawings were adapted from Paxinos and Watson (1998).

damage to anterior portions of temporal association cortex (area TE) and auditory association cortex in all rats. One PRh rat sustained minor unilateral damage to the anterior portion of the postrhinal cortex, and another rat sustained minor unilateral damage to the ventral CA1 subfield.

4.3.3. Behavioural results

4.3.3.1. mDNMS task

4.3.3.1.1. Post-surgery training stage 1. All rats, except for one PRh rat, reached the performance criterion within the allotted 50 sessions. Thus, the data for this rat were excluded from the analyses. Figure 4.3a depicts the mean percent correct choices on the first and last five sessions for each group. A One-way ANOVA revealed no significant difference between groups on the mean percent correct choices on the last five sessions, ($F_{(2,12)} = .43$, $p = .66$, $\eta^2 = .08$). Figure 4.3d depicts the mean number of sessions each group required to reach the performance criterion (excluding criterion sessions). A One-way ANOVA revealed no significant group difference ($F_{(2,12)} = 3.06$, $p = .09$, $\eta^2 = .38$).

4.3.3.1.2. Post-surgery training stage 2. Figure 4.3b depicts the mean percent correct choices on the first and last five sessions for each group. One HPC rat failed to reach the performance criterion within 50 sessions, and the data for this rat were excluded from the analyses. A Levene's test for equality of variances was found to be violated for the mean percent correct choices on this stage, thus a nonparametric test was performed. A Kruskal-Wallis H test revealed that there was no significant difference in mean percent correct choices on the last five sessions, χ^2 (2) = 2.67, $p = .26$. Figure 4.3e depicts the mean number of sessions each group required to reach the performance criterion (excluding criterion sessions). A One-way ANOVA revealed no significant group difference ($F_{(2,12)} = 1.59$, $p = .25$, $\eta^2 = .24$).

4.3.3.1.3. Post-surgery training stage 3. Figure 4.3c depicts the mean percent correct choices on the first and last five sessions for each group. A Kruskal-Wallis H test revealed that there was no significant difference in mean percent correct choices on the last five sessions, χ^2 (2) = 1.25, $p = .54$. Figure 4.3f depicts the mean number of sessions each group required to reach the performance criterion (excluding criterion sessions). A Kruskal-Wallis H test revealed that there was no significant difference in mean number of sessions required to reach criterion, χ^2 (2) = 1.85, $p > .05$.

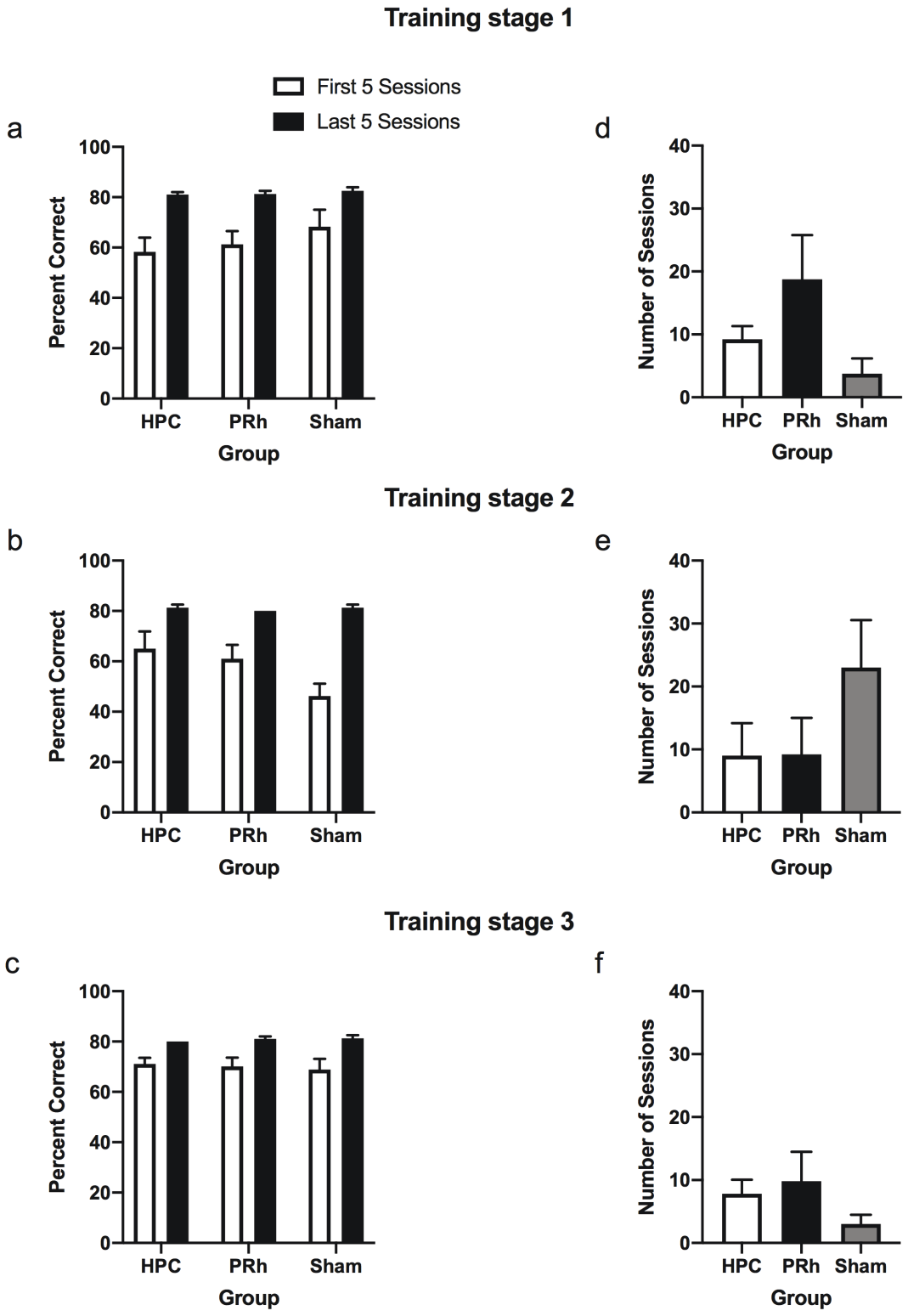

Figure 4.3. (a-c) Average scores (± SEM) on the first and last five sessions and (d-f) mean (± SEM) number of sessions required to reach the performance criterion for each group on training stage 1, 2, and 3, respectively.

4.3.3.1.4. Acquisition of mDNMS task. Figure 4.4a depicts the mean percent correct choices on the first and last five sessions for each group. All rats acquired the nonmatching rule, except for one HPC rat, and the data for this rat were excluded from the analyses. The closest this rat came to reaching the performance criterion was 75% on five consecutive sessions by Session 48. A Kruskal-Wallis H test revealed that there was no significant difference in mean percent correct choices on the last five sessions, χ^2 (2) = 1.08, p = .58. Figure 4.4b depicts the mean number of sessions each group required to reach the performance criterion (excluding criterion sessions). A One-way ANOVA revealed no significant difference in mean number of sessions to reach criterion ($F_{(2,12)}$ = 0.54, p = .60, η^2 = .10).

4.3.3.1.5. Probe tests. One PRh rat became ill and was euthanized prior to the probe tests, so the results for the PRh group are based on four rats. Moreover, the one HPC rat that did not reach criterion was excluded from the probe test analyses.

Figure 4.5 depicts the results from the probe tests. We compared each group's scores on the probe tests to chance level of performance using a one-sample t-test (one-tailed). Rats in the HPC group had scores that were significantly different from chance on the "No Reward" probe ($t_{(3)}$ = 3.06, p = .03, Hedge's g = 2.09, 95% CI [0.31, 4.01]). However, the HPC group scores were not significantly different from chance on the "Sample-Baited" probe ($t_{(3)}$ = -1.42, p = .13, Hedge's g = -0.96, 95% CI [-2.45, 0.53]) or the normal tests ($t_{(3)}$ = 1.00, p = .20, Hedge's g = 0.94, 95% CI [-0.76, 2.12]). Rats in the PRh group did not have scores significantly different from chance on the "No Reward" probe ($t_{(3)}$ = .71, p = .27, Hedge's g = 0.5, 95% CI [-0.91, 1.92]) or the "Sample-Baited" probe ($t_{(3)}$ = 0.00, p = .50, Hedge's g = 0.0, 95% CI [-1.39, 1.39]). Moreover, the PRh rats' scores on the normal tests were also not significantly greater than chance ($t_{(3)}$ = .78, p = .25, Hedge's g = 0.55, 95% CI [-0.89, 1.94]). The Sham group had scores that were significantly above chance on the "No Reward" probe ($t_{(3)}$ = 3.57, p = .02, Hedge's g = 2.52, 95% CI [0.53, 4.50]), the "Sample-Baited" probe ($t_{(3)}$ = 7.00, p = .003, Hedge's g = 4.98, 95% CI [1.84, 8.12]), and on the normal tests ($t_{(3)}$ = 3.00, p = .03, Hedge's g = 2.15, 95% CI [0.31, 3.99]).

Separate one-way ANOVAs were performed to determine if there was a significant difference in scores between the probe tests and normal tests for each group of rats. There was a significant difference between scores on the probe tests and normal tests for the HPC group ($F_{(2,11)}$ = 4.53, p = .04, η^2 = .50). Post hoc comparisons (Bonferroni corrected) revealed a

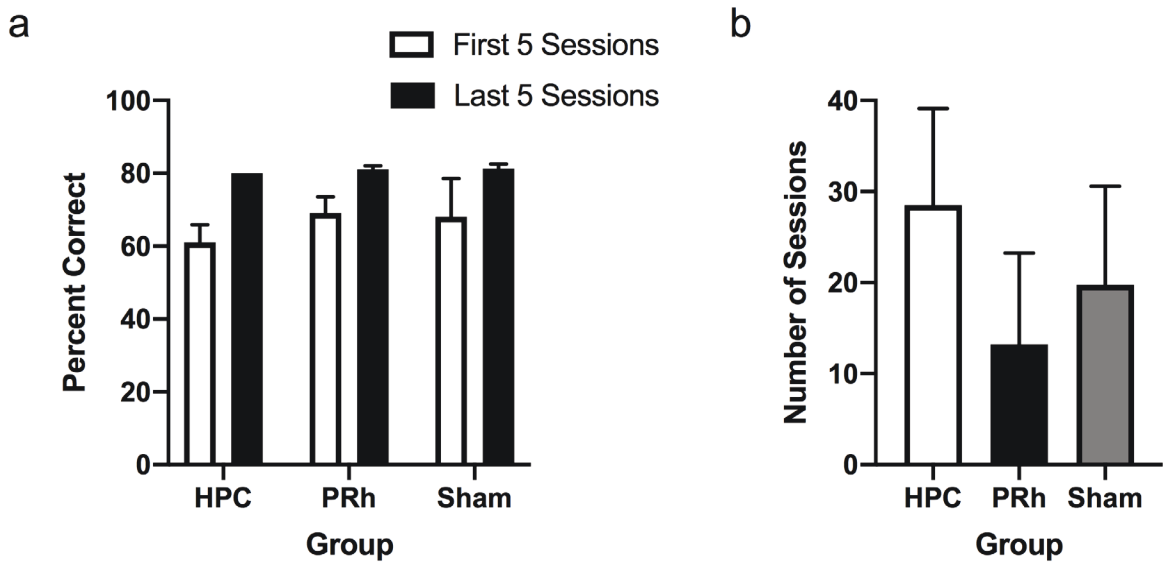

Figure 4.4. (a) Average scores (± SEM) for each group on the first and last five sessions of the mDNMS acquisition and (b) mean number of sessions required to reach the performance criterion.

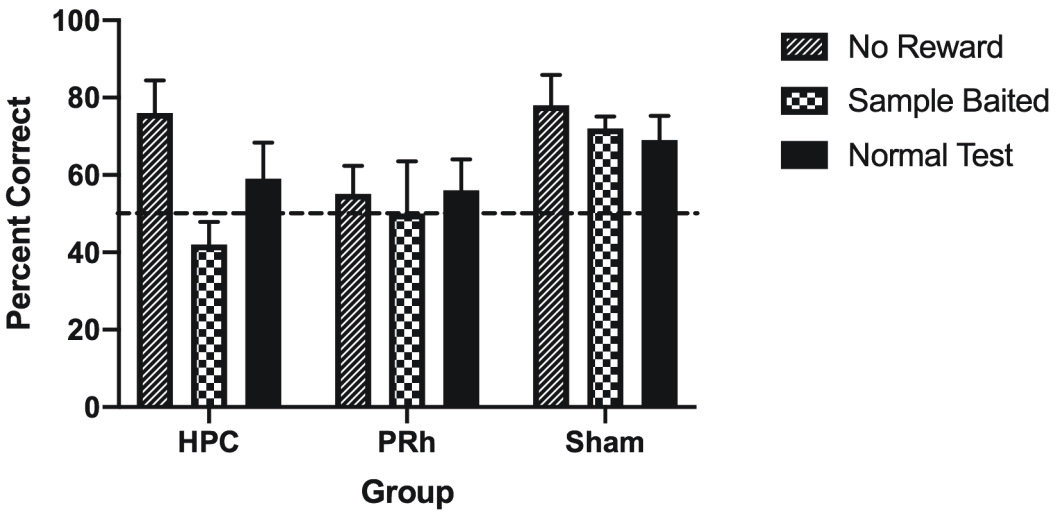

Figure 4.5. Average scores (± SEM) on the probe and normal test trials for each group. Dashed line represents chance performance.

significant difference between the "No Reward" and "Sample-Baited" probe scores (p = .04), however, scores on the probe tests did not significantly differ from those on the normal tests. There was no significant difference between the probe test scores and normal test scores for the PRh group ($F_{(2, 11)}$ = .11, p = .90, η^2 = .02) or for the Sham group ($F_{(2,11)}$ = .62, p = .56, η^2 = .12).

4.3.3.2. NOP test. Figure 4.6a depicts the total time spent investigating objects during the 5-min familiarization phase for each group. A One-way ANOVA revealed no significant difference between groups ($F_{(2,12)}$ = 2.17, p > .05, η^2 = .30). Figure 4.6b depicts the mean investigation ratios on the test, which are based on the first 3 cumulative minutes. One-sample t-tests (one-tailed) revealed that the HPC group had a mean investigation ratio significantly above chance level of performance ($t_{(4)}$ = 2.77, p = .03, Hedge's g = 1.74, 95% CI [0.04, 3.44]). Conversely, both the PRh group and Sham group had investigation ratios that were not significantly different from chance level of performance ($t_{(3)}$ = -.54, p > .05, Hedge's g =.35, 95% CI [-1.75, 1.05]) and ($t_{(3)}$ = .52, p > .05, Hedge's g =.38, 95% CI [-1.02, 1.79]), respectively. Both the Sham and PRh group never had investigation ratios significantly above chance, whereas the HPC group continued to have scores significantly above chance in the first 4- and 5-cumulative minutes of the test.

4.3.3.3. Correlations. A Pearson correlation was performed to determine whether there was a relationship between the extent of HPC or PRh damage and the mean percent correct choice during the five criterion sessions on the mDNMS acquisition stage. For the HPC rats, the correlation coefficient was not statistically significant (r = -0.51, p = .38, 95% CI [-.68, .96]), nor was it statistically significant for the PRh rats, (r = -0.85, p = .07, 95% CI [-.13, .99]). Moreover, a Pearson correlation was performed to determine whether there was a relationship between the extent of HPC or PRh damage and mean number of sessions required to reach the performance criterion. The correlation coefficient was not statistically significant for either the HPC group (r = -.05, p = .94, 95% CI [-.90, .87]) or the PRh group (r = -.10, p = .87, 95% CI [-.90, .86]).

4.4. Discussion

Rats with PRh lesions, and all but one HPC rat, successfully reached the performance criterion on the mDNMS task. Compared to Sham rats, the PRh and HPC groups reached a similar level of accuracy on the test and required a similar number of sessions to reach the

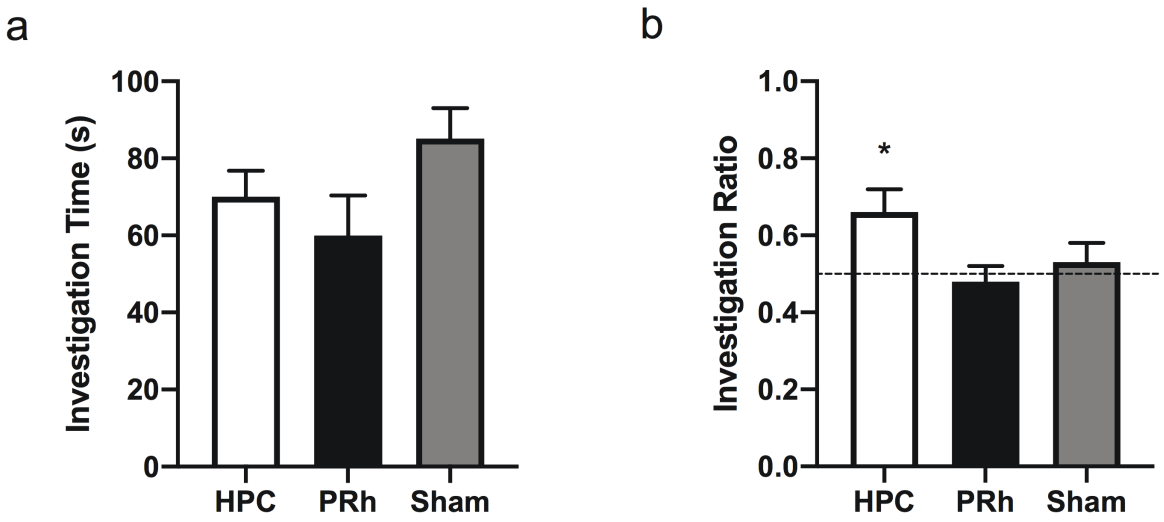

Figure 4.6. (a) Average (± SEM) time spent investigating objects during the familiarization phase of the three NOP trials. (b) Mean (± SEM) investigation ratios during the first 3 min on the NOP test for each group. Asterisk denotes a group mean that is significantly above chance level (one- sample t-test, $p < .05$).

performance criterion. The HPC rat that failed to reach criterion came close, reaching an accuracy of 75% on five consecutive sessions. Thus, overall, rats with either HPC or PRh lesions were capable of retaining information for four sample objects over a short retention interval on the mDNMS task.

Consistent with previous DNMS findings, HPC rats acquired the mDNMS task at the same rate as control rats (Aggleton et al., 1986; Clark et al., 2001; Mumby, Pinel, Kornecook, Shen, & Redila, 1995; Shaw & Aggleton, 1993). Although the HPC rats' scores during mDNMS acquisition were not significantly different from Sham rats, there was one HPC rat that failed to reach criterion within 60 sessions. The reason why this rat failed to reach the criterion cannot be determined from the available data. However, previous research has shown that DNMS performance deficits can be more severe when rats with HPC lesions do not receive pre-surgery training (for a review see Mumby, 2001). This suggests that an intact HPC is important for acquiring other skills that are important for successful performance on the task (e.g., avoiding distractions during the delay or withholding hasty responding). An advantage of providing rats with extensive DNMS training prior to surgery is that it can help rule out other potential reasons for impaired performance. Accordingly, to further examine this question, an important next step would be to first train rats on all stages of the mDNMS task and then administer HPC lesions. Regardless, four out of five HPC rats successfully reached the performance criterion, and did so following a similar number of trials compared to the Sham group. It is important to note that the rats in the present experiment received some pre-surgery training on procedural aspects of the task. The extent to which this pre-surgery training facilitated the acquisition of the task is unclear. Accordingly, to compare mDNMS task acquisition to that on conventional DNMS tasks, it would be important to conduct an experiment whereby no pre-surgery training is administered. In any case, the present findings reveal that overall, rats with HPC lesions successfully discriminated between familiar and unfamiliar objects on the mDNMS task.

The mDNMS results for the PRh group were not in-line with the hypothesis that the PRh plays a critical role in object-recognition memory. Based on this hypothesis, we predicted that rats with PRh lesions would require significantly more trials to reach criterion compared to Sham rats. There was no significant difference, however. Previous rodent DNMS studies found that rats with PRh lesions performed significantly worse than control rats following delays lasting 15 s or longer (Mumby & Pinel, 1994; Wiig & Bilkey, 1995). Moreover, Wiig and Bilkey (1995)

found that PRh rats required more trials to reach the performance criterion on the DNMS compared to Sham rats. Comparatively, the PRh rats in the present experiment successfully reached the performance criterion despite using a longer retention interval (26 vs. 4 s) and the presentation of *four* distinct sample objects rather than one sample object. Thus, PRh rats in the present experiment were capable of retaining more item information over a longer delay relative to previous studies. Consequently, rats' performance on the mDNMS task appears to be more robust compared to conventional DNMS tasks. However, as previously stated, rats were provided with some pre-surgery training, therefore it is possible that such training may have facilitated the acquisition of certain skills necessary for normal performance on the task.

One PRh rat did fail to reach the performance criterion within the allotted number of sessions during training stage 1, despite the initial pre-surgery training. However, by training stage 2 this rat successfully reached the criterion and continued to do so on subsequent stages, revealing it was capable of learning the nonmatching rule and discriminating between sample and novel objects. Overall, the mDNMS results indicate that the rats with PRh lesions were capable of recognizing sample objects following a brief retention interval of around 3 minutes.[9]

Rats with HPC lesions exhibited significant novel-object preferences on the NOP test, indicating they recognized the sample objects on the test. Thus, consistent with previous findings, the HPC does not appear to play a critical role in the ability to detect the familiarity of a previously encountered object (see Cohen & Stackman, 2015 for a review; Ennaceur & Aggleton, 1994; Ennaceur et al., 1996; Ennaceur & Aggleton, 1997; Forwood, Winters, & Bussey, 2005; Gaskin, Tremblay, & Mumby, 2003; Mumby, Gaskin, Glenn, Schramek, & Lehmann, 2002; Winters et al., 2004). Conversely, both the PRh and Sham group failed to display a novel-object preference on the test. The NOP results for the PRh group are consistent with the notion that PRh damage produces object-recognition impairments, however, this does not explain the same PRh rats' successful performance on the mDNMS task. The inconsistencies in results on the two tests are a reflection of the internal-validity problem with the NOP test, especially when a failure to discriminate is taken as evidence of a failure to recognize objects. Accordingly, we propose that object-recognition abilities were largely spared in rats with PRh lesions, and their failure to show a novelty preference on the NOP test is due to some other

[9] In fact, the average delay length between the moment a rat stopped investigating one sample object on the sample phase and the corresponding test trial for that sample object on the mDNMS task was ~180 s.

unidentified behavioural, cognitive, or motivational impairment. Indeed, there are a few reasons to question whether this lack of a novel-object preference in the PRh group reflects an object-recognition deficit. First, the same rats showed intact memory for four sample objects on the mDNMS task following a similar delay. It seems unlikely that a PRh lesion would disrupt the ability to remember one sample object over a 180-s delay on the NOP test without causing an impairment in the ability to remember *four* objects over a similar delay. Moreover, the fact that the control rats also failed to exhibit a novel-object preference demonstrates that one should be cautious interpreting a lack of novel-object preference as indicative of an object-recognition memory impairment. Indeed, the NOP test has been described as being advantageous over DNMS paradigms when interpreting treatment effects because it reduces interpretational problems that may arise unintentionally due to: 1) reference-memory impairments, such as failing to learn the nonmatching rule and 2) disruptions in non-mnemonic processes, such as motivational responses to a reinforcer (i.e., food reward) (Antunes & Biala, 2012; Clark & Martin, 2005; Clark & Squire, 2010; Cohen & Stackman, 2015; Ennaceur & Delacour, 1988; Hughes, 2007; Silvers, Harrod, Mactutus, & Booze, 2007; Winters, Saksida, & Bussey, 2008). However, a factor that is overlooked is the possibility that a treatment may affect non-mnemonic processes related to the behavioural expression of this novelty preference. Indeed, the number of potential reasons for why a rat fails to display a novel-object preference on the test is confounded by the fact that the NOP test does not involve a goal, and thus the rat is not required to make an explicit choice response based on memory. Alternatively, it is possible that the PRh and Sham group NOP results reflect low sample sizes, as typically a larger sample is required when using the NOP test due to the variability in object investigation. However, the lack of statistical significance on the test does not appear to be due to high levels of variability, as the variability between scores in both the PRh and Sham groups was considerably low (see Figure 4.6b). Regardless, the results from the present study reveal that interpreting the effects of a treatment on object-recognition using the NOP test is not straightforward, and one should exert caution when proposing reasons for null preferences on the test.

A reconciliation of the divergent effects of PRh lesions on rats' performance on the mDNMS task and NOP test may lie in differences in how object representations are encoded or retrieved during appetitively-motivated tasks, such as the DNMS task, and incidental learning tasks, such as the NOP test. Nonhuman primates with HPC damage, for example, have failed to

exhibit novelty preferences on the *visual paired comparison* (VPC) task (an analogue of the rodent NOP test) despite attaining high accuracy scores on the DNMS task (Nemanic et al., 2004; Pascalis & Bachevalier, 1999). The authors interpreted the lack of novelty preference on the VPC task as an object-recognition memory impairment, which led them to conclude that the way in which a stimulus is encoded (either actively or passively) potentially requires different brain regions (Nemanic et al., 2004). Consequently, damage to a particular brain area can disrupt memory for objects on one task but not the other. While this interpretation may explain the results for the PRh rats in the present experiment, it fails to explain why we also observed the same divergent results between tasks for the Sham group. Moreover, it fails to explain why PRh lesions do not consistently disrupt novel-object preference in rats.

A more likely explanation for the divergent effects of PRh lesions on rats' performance on both tasks is that both tasks do measure the ability to detect the familiarity of a previously encountered object, yet they differ in their precision to do so. On DNMS tasks, rats are trained to learn a nonmatching rule, thus with continued testing, on the sample phase the rat presumably actively memorizes features of the sample object in order to successfully select a future response to retrieve a food reward (nonmatch-to-sample). Displacing the object from over the food well and learning that the object features (size, texture, and shape) are integral for successful performance on the test, may make it such that the rat focuses more on encoding stimulus features. Conversely, on the NOP test, the rat incidentally explores objects in an environment. On the familiarization phase the rat is encoding information not only about the sample objects but also their location relative to spatial cues and the surrounding context. On the test rats may rely on spatial and nonspatial information to successfully discriminate between the sample and novel object. Thus, when a rat fails to display a novel-object preference following some treatment, it could reflect a disruption in memory for a number of factors related to information learned during the familiarization phase. For example, the treatment may have disrupted memory for the sample object, the particular context in which the object was encountered (e.g., room and apparatus), or even the specific location of the sample object relative to cues within the apparatus (including the second copy of the sample object). This raises concerns about potential confounds that are introduced when trying to measure a rat's ability to detect the familiarity of a previously encountered object when it is not required to make an explicit choice-response based on memory

for the sample object. Indeed, this may explain the inconsistent findings across NOP studies following some treatment.

Interestingly, rats with PRh lesions fail to display novelty preferences on the object-in-place variant of the NOP test (Barker et al., 2007; Bussey et al., 2000; Liu & Bilkey, 2001). On the object-in-place task, a rat is familiarization to four distinct sample objects—one in each corner of a square open-field arena. Then, on the test, two of the objects swap places. Intact object-in-place memory is inferred when a rat spends more time investigating the two objects that switched places relative to the two that remained in the same place. Considering normal rats detect this change, it suggests that while a rat is in the open field, it acquires information about a particular object and its location relative to spatial cues, which include the other objects in the open field. With that in mind, given that rats with PRh lesions fail to spend more time investigating the objects that swap compared to those that remain in place, it is not implausible to consider that a lack of novelty preference on the standard NOP test may reflect a similar disruption in a rat's ability to detect changes made to the spatial cues surrounding the sample object (i.e., replacing the second sample object with a new cue). Depending on what a particular rat attends to during the familiarization phase, this could explain why there are discrepant findings on the effects of PRh lesions using the NOP test. In any event, these inconsistent findings highlight the advantages of designing a task that incorporates the use of an instrumental response with which the rat is required to make an explicit choice between familiar and unfamiliar objects, and one that includes rewards for accurate choices.

Other researchers have reported that under certain experimental conditions, rats with PRh lesions are capable of both detecting the familiarity of an object and displaying a bias to investigate novel objects more than familiar ones (Albasser et al., 2011, 2015; Olarte-Sánchez, Amin, Warburton, & Aggleton, 2015). When PRh lesion rats were presented with object pairs using the standard NOP test method –one novel and one familiar ("novel + familiar")— they failed to show an investigatory bias towards the novel object. Interestingly, when the same rats were given several consecutive tests that contained either a pair of novel objects ("novel + novel") or a pair of familiar objects ("familiar + familiar"), they spent more time investigating the novel-object pairs compared to the familiar-object pairs. The difference in time spent investigating familiar and novel object pairs suggests that the PRh rats recognized the familiar stimuli. Overall, the authors concluded that PRh lesions might disrupt rats' ability to discriminate

which particular object is novel or familiar when both objects are presented simultaneously (Olarte-Sánchez et al., 2015). This hypothesis is in line with the notion that rats rely on both spatial and nonspatial information to successfully discriminate between the novel and sample object on the NOP test, and that the PRh may play a role in the ability to detect the specific location of an object relative to local cues. The divergent results between the object-pair presentation conditions for the PRh rats could be explained based on changes to the spatial information between the learning and test phase. On trials in which a pair of familiar objects was presented to the rats, both the spatial and nonspatial information between the learning and test phase remained the same. However, on the trials in which a novel and familiar object were presented simultaneously, the spatial information changed between the learning and test phase (i.e., the spatial relation between the sample and novel object was new). Further experiments are required to determine if the PRh plays a critical role in the ability to remember the specific location in which an object was previously encountered.

The results from the probe tests revealed that Sham rats' had scores that were significantly above chance on both types of probe test and normal tests. Moreover, there was no significant difference in the Sham rats' performance on the probe tests and normal tests. This indicates that they were not relying on olfactory cues to correctly locate the food reward buried underneath the novel object during testing. Conversely, PRh rats' scores on both types of probe tests and the normal tests were not significantly different from chance. The low scores on the normal tests make it difficult to interpret the probe test results. The fact that PRh rats did not show a bias to displace the novel objects first on the normal tests, or the sample objects first on the "Sample-Baited" probes suggests they were not relying on olfactory cues to correctly locate the food reward. In the experiment presented in Chapter 2, we reported that when administering probe tests, it modifies the characteristics on the task, which consequently, can disrupt performance on the test despite intact recognition abilities (Honig & Dodd, 1986). We suspect that introducing these changes to the reward contingency during probe testing disrupted PRh rats' performance on both the normal tests and probe tests. Accordingly, the decline in task accuracy during probe testing may have reflected the rat's incentive to respond accurately, and not necessarily memory abilities or the ability to detect the odor of the reward. The HPC rats' scores on the normal tests were not significantly different from chance. However, their scores on the "No Reward" probe tests were significantly above chance, indicating that they accurately

selected the novel object first when no food reward was present. This latter finding suggests that HPC rats were not relying on olfactory cues to correctly locate the food reward buried beneath the novel object during previous testing. The HPC rats' scores on the "Sample-Baited" probes were not significantly different from chance, although there was a slight trend for the HPC rats to select sample objects first on the "Sample-Baited" probe tests. Upon closer inspection of their scores on each test trial within the "Sample-Baited" probe sessions, it revealed a tendency for the HPC rats to displace the novel object first on test trial 1 (mean accuracy score was 75%), and the sample object first on test trial 2, 3, and 4 (mean accuracy scores were 25%, 38%, and 38%, respectively). Thus, on the "Sample-Baited" probe sessions, it appeared as though the HPC rats quickly applied the new information that was learned on test trial 1 to the subsequent tests within that session (i.e., the sample is now rewarded). Thus, the HPC rats' performance on the "Sample-Baited" probe tests does not appear to demonstrate the use of olfactory cues to correctly locate the food reward, rather it appears to reflect the ability to apply new information learned on one trial to the subsequent trials. Overall, the probe tests results suggest that rats were not relying on olfactory cues to successfully locate the food reward buried beneath the novel object on the test. However, administering probe tests can disrupt normal performance on a task, and the probe tests administered in this experiment appeared to especially be disruptive to PRh rats' performance.

The extent of damage made to the PRh cannot explain the lack of impairment on the mDNMS task, as the majority of the PRh was removed bilaterally in all of the rats. Moreover, it is difficult to argue that the PRh lesions were not complete enough to disrupt performance as the size of the lesion was similar to those in previous experiments that found impaired object-recognition abilities after PRh lesions in rats (Mumby & Pinel, 1994; Wiig & Bilkey, 1995). The PRh rats in the present experiment, however, received moderate damage to area Te2, which has strong reciprocal connections with the PRh and provides visual information to it (Burwell & Amaral, 1998). This unintentional damage to area Te2 may explain the lack of novel-object preference on the NOP test. Previous research using the NOP test has shown that rats with combined lesions to the PRh and area Te2 fail to exhibit a novel-object preference following a 15-min delay (Aggleton et al., 1997). However, this effect does not appear to reflect the damage made to area Te2 because excitotoxic lesions restricted to area Te2 have failed to disrupt novel-object preferences following delays lasting 5 min (Weng-Thim Ho et al., 2011). Thus, it seems

unlikely that the resulting NOP deficit reflects damage to area Te2, however, we cannot rule out the possibility that the unintentional damage contributed to the lack of novel-object preference. We can confirm, based on the mDNMS task results, that both perceptual and object-recognition abilities remained intact in rats with PRh lesions.

In summary, rats with either HPC or PRh lesions successfully acquired the mDNMS task and reached accuracy scores that were comparable to Sham rats. Thus, rats with either HPC or PRh lesions were capable of retaining information for four sample objects over a short retention interval. The reason why rats in the PRh and Sham group failed to show a novel-object preference on the NOP test cannot be determined from the available data. However, the successful performance on the mDNMS task for both groups suggests that the lack of a novel-object preference on the NOP test did not reflect an object-recognition memory impairment. In the present experiment, memory for objects was assessed only following short retention intervals. Accordingly, an important next step is determining the effects of PRh lesions on rats' ability to perform the mDNMS task following retention intervals lasting longer than several minutes. This would be essential in order to confirm the robustness of the mDNMS task compared to conventional DNMS tasks. Collectively, the findings from the present experiment reveal that it is difficult to make firm conclusions about the status of object-recognition memory using the NOP test, and what is needed, is a task that provides less ambiguous interpretations of a rat's behaviour on the test, one such as the mDNMS task.

Chapter 5: A Go/No-go delayed nonmatching-to-sample procedure to measure object-recognition memory in rats

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Abstract

The *modified delayed nonmatching-to-sample* (mDNMS) task has advantages over existing object-recognition tests in that it is easier to train rats on compared to conventional DNMS procedures, and interpreting recognition abilities on it is less ambiguous than on the *novel-object-preference* (NOP) test. Despite these improvements, there are still features of the mDNMS task that can be improved on. Accordingly, the goal of this experiment was to refine the mDNMS task. We decided to incorporate a *Go/No-go* procedure that had the added benefit of introducing an additional dependent measure to assess object-recognition memory in rats: *latency to displace objects*. Rats received sessions whereby they were trained to approach and displace an unfamiliar object (sample) from over a food well to obtain a food reward (sample phase). Then on a choice phase, rats received trials in which either a copy of the sample object or a novel object was presented. On trials in which a novel object was presented ("Go" trial), the rat received a reward for approaching and displacing it. Conversely, on trials in which a sample object was presented ("No-go" trial) the rat was not rewarded for displacing it and was trained to withhold responding. We measured both latency to displace objects and response accuracy (a correct choice was defined as either displacing a novel object or not displacing the sample). Rats required an average 54 sessions to reach a performance criterion of at least 80% correct choices on five consecutive sessions (16 correct choices out of 20). Moreover, rats displayed significantly longer latencies to displace objects on No-go trials compared to Go-trials on five consecutive sessions following an average of 36 sessions. Both results indicate that the rats acquired the reward contingency (a novel object predicts food). After rats acquired the Go/No-go DNMS task, we tested them on the NOP test to determine if scores on both tasks were significantly correlated. We found no significant correlation, indicating that performance on the Go/No-go DNMS task did not predict novelty-preference scores. The findings from this experiment highlight the benefits of incorporating multiple dependent measures to assess object-recognition memory. Moreover, compared to the NOP test, the features of the Go/No-go DNMS task allow for a more straightforward interpretation of behaviour in relation to object-recognition memory.

5.1. Introduction

Animals can exhibit performance impairments on memory tests for reasons that are unrelated to mnemonic functions. Interpreting the cause for a performance deficit can be a challenge if potential extraneous variables are not controlled for. Accordingly, a key feature of a robust research design is the reduction or elimination of measurement error. We recently developed a modified delayed nonmatching-to-sample (mDNMS) task that is an improvement on conventional DNMS tasks in that rats require significantly fewer trials to reach the performance criterion and it can be used to assess long-term object-recognition memory (Cole, Simundic, Mossa, & Mumby, 2019). Moreover, compared to the novel-object-preference (NOP) test it provides a less ambiguous measure of the status of object-recognition memory. Specifically, it incorporates the use of an instrumental response with which the rat makes an explicit choice between familiar and unfamiliar objects, and rats are provided with a reward for accurate choices. Despite these improvements, there may yet be opportunity to further improve the procedure. Accordingly, the aim of the present experiment was to modify certain features of the mDNMS task that have the potential to interfere with accurate performance on the test.

One drawback associated with DNMS tasks is that rats can develop positional biases on the test (i.e., consistently displacing an object based on its spatial location—left or right—and not whether it is novel). This behaviour is disruptive and if not caught, interferes with accurate performance on the test (cf. Kalynchuk & Beck, 1992; Krechevsky, 1932; Mumby, 2005; Mumby, Pinel, & Wood, 1990; Rothblat & Hayes, 1987). These positional biases can form either early on during DNMS training, prior to when the rat learns the reward contingency (Rothblat & Hayes, 1987) or following the introduction of longer retention intervals (Mumby et al., 1990). In the latter instance, it may reflect a strategy that a rat resorts to when it fails to remember the sample object due to the increase in demands on memory (Mumby et al., 1990). In both cases, additional training is typically provided in an attempt to remove these positional biases. The standard approach involves rewarding the non-preferred side more than the preferred side (e.g., using a ratio of 9:1) until the behaviour ceases. If the experimenter is unable to remove these biases, then the rat is removed from the experiment. Indeed, several rats were excluded from the Cole et al. (2019) experiment because they developed positional biases on the test. Whether positional biases are formed early on in training or after introducing long retention intervals, they are disruptive and ultimately can lead to the exclusion of rats or trials from the experiment. Thus,

a major objective in the present experiment was to modify the mDNMS task to prevent the formation of positional biases.

Moreover, although the rats required significantly fewer trials to reach a strict performance criterion on the mDNMS task compared to conventional DNMS tasks, accuracy scores on the shortest delay declined significantly between the five criterion sessions during training and subsequent mixed-delay testing. Mean accuracy scores on the five criterion sessions in the experiments from Chapter 2 and 3 were 84% and 81%, respectively. Thereafter, scores on the shortest delay during mixed-delay testing dropped to 68% and 65%, respectively. This is a problem because a key requirement for any DNMS task is that normal rats maintain high accuracies on the test, as it increases the ability to detect even slight impairments in performance following some form of treatment (e.g., surgical lesion). As described in Chapter 2, we suspected that the discrepancy in scores between training and testing likely reflected an effect of the different testing procedures on *performance* more so than on memory. Alternatively, it could reflect the increase in the length of the minimum delay used during training and testing (e.g., the delay increased from 30 to 100 s). Thus, the decline in accuracy may reflect an increase in the demands on memory as the delay length became longer. Neither of the aforementioned explanations, however, can account for the observed decline in performance in the experiment presented in Chapter 3. In that experiment there was no significant increase in the minimum delay length between training and testing (69 to 82 s) nor were there abrupt changes to the delay length inside the apparatus.

We posited that the reduced accuracy on the test might reflect the low cost to making an error on the test. Unlike conventional DNMS tasks, on the mDNMS task the rat has the opportunity to obtain a food reward after making an initial error, as the food reward is placed underneath the novel object prior to the start of the trial. On conventional DNMS tasks, during the choice phase the subject receives a reward *only* if it displaces the correct stimulus (novel object) and inhibits responding to the incorrect stimulus (sample object). For example, when the subject displaces the sample object on the test, the experimenter withholds the food reward and abruptly ends the trial by removing both objects from the apparatus. Providing the opportunity to make a second choice on the mDNMS task may have facilitated acquisition of the nonmatching rule, however, doing so for extended periods of testing may have been detrimental for performance. Theoretically, allowing the rat to make a second choice on each trial would

increase task-acquisition rate because it provides more opportunities for the rat to learn that the novel object predicts a food reward and the sample object does not (instead of the rat only learning one part of the reward contingency on each trial). However, over time there may have been a reduction in demand to make accurate first choices on the test as the rat learned it did not require much time and/or energy to displace the novel object after an initial incorrect response. Although we increased both the delay and amount of effort necessary to retrieve the buried Cheerio from beneath the novel object, it may not have rendered enough of a cost to deter rats from displacing the sample object before the novel. This feature of the task may explain the observed decline in accuracy as testing continued, despite the rats requiring significantly fewer trials to reach the performance criterion compared to conventional DNMS tasks (however, see Appendix B for an alternative explanation).

In order to refine the mDNMS paradigm to minimize the abovementioned issues, the following changes were required: 1) removing the potential for positional biases to form, and 2) increasing the cost for making an error. In making the adjustments, we thought it was essential that the rats continued to "self-administer" trials. First, removing the experimenter from playing an active role in administering individual trials removes other extraneous factors that could influence a rat's performance on the test (e.g., distraction due to the presence of the experimenter or the experimenter unintentionally giving cues to the rat as to which object will be rewarded on the choice test). Second, having rats travel at their own pace around a large environment reflected a more ecologically valid paradigm compared to conventional DNMS apparatuses. Indeed, the design of the apparatus more closely mimicked how a rat naturally forages in the wild –visiting new locations and avoiding previously visited ones (Barnett, 2005). Accordingly, one way to implement the changes while using the same apparatus would be to increase the distance between the sample and novel objects on the test. By placing only one object (either a sample or novel) on each test level of the apparatus, the objects would no longer be positioned next to each other thus, removing the potential for positional biases to form. Additionally, by increasing the distance between the sample and novel objects, a rat could no longer displace a sample object and then quickly retrieve the reward from beneath an adjacent novel object. By increasing the distance required to visit each object on the test, it would increase the cost for making an error (i.e., displacing the sample object). Theoretically, this should reduce a rat's

tendency to displace sample objects on the test, and consequently result in higher accuracy scores on the test.

In implementing these changes, we essentially developed a *Go/No-go DNMS* procedure to measure object-recognition memory. Rats were trained to approach and displace an unfamiliar object (the sample) from over a food well to obtain a food reward. Then, rats received a choice phase whereby they were presented with copies of the sample object on two test levels and novel objects on the remaining two levels. On trials in which the novel object was presented ("Go" trial), the rat received a reward for approaching and displacing it. On trials in which sample objects were presented ("No-go" trial) rats were not rewarded and were trained to withhold responding (do not displace the object). Notably, the new procedure made it possible to use two dependent measures as an index of object-recognition memory: *percent correct choices* and *latency to displace an object*. A correct choice was defined as either displacing a novel object or not displacing a sample object. Thus, a rat could display two different types of behaviours, each of which could be used to determine whether the rat successfully discriminated between a familiar and unfamiliar object.

A secondary goal of this experiment was to determine whether performance on the Go/No-go DNMS task predicted novel-object preference scores on the NOP test. If novel-object preference scores provide an accurate estimate of a rat's object-recognition abilities, then we predicted to find a positive linear correlation to exist between scores on the Go/No-go DNMS and NOP test. Accordingly, after rats acquired the Go/No-go DNMS task we tested them on the NOP test.

5.2. Materials and Method

5.2.1. Subjects

The subjects were ten male Long-Evans rats (Charles River, Kingston, ON), 450-550 g at the start of mDNMS training (~21 weeks old). The rats were pair-housed in polypropylene cages (48 × 25 × 20 cm) in a colony room maintained under a reverse 12:12 light-dark cycle, with light onset at 8:00 p.m. The rats had continuous access to water and each received a daily ration of ~25 g of rat chow (Charles River Rodent Animal Diet, no. 5075) in the late afternoon, after behavioral testing was finished for the day. Prior to the start of the experiment, the rats received 14 weeks of environmental enrichment starting on post-natal day 28. Environmental enrichment entailed placing all 10 rats in a large apparatus, similar to the one used in the present experiment,

for five hr/day, five days/week. During environmental enrichment, the rats were exposed to different events as part of an unrelated experiment (e.g., forage for novel foods, and on occasion encounter aversive stimuli (cat collar infused with cat odour and a lithium chloride injection following the ingestion of a novel food). Moreover, the rats encountered stimulus objects, but none of which were used in the present experiment. Following enrichment the rats were used in a series of brief unrelated experiments involving exposure to aversive stimuli (e.g., receiving a foot-shock in a conditioning chamber or being placed in a water maze). The training histories were identical for all rats in the present experiment. All procedures were approved by the Concordia University Animal Care and Use Committee, and were in accordance with the guidelines of the Canadian Council on Animal Care.

5.2.2. Apparatuses

5.2.2.1. Go/No-go DNMS task. The same large multi-level apparatus (152 × 145 × 86 cm) that was used for mDNMS testing was used in the present experiment (Figure 5.1). The apparatus was a modified, freestanding steel cage rack, enclosed on three sides by wire mesh, with a removable, clear acrylic front panel. The apparatus had five levels, each covered with woodchip. The top four levels were divided into two equal halves by a plastic barrier wall, and the bottom level remained undivided. A loading cage (58 × 37 × 20 cm) was placed on the top left side of the apparatus. A rat entered the apparatus via a hole in the bottom of the loading cage that was placed over a passageway leading to the top level of the apparatus. Rats traversed the different levels via wire mesh passageways located on both sides of the apparatus. The design of the apparatus was such that a rat had to climb down the passageways on the left side of the apparatus in order to gain access to the right side, which it then could ascend from level to level. The top four divided levels each contained one plastic rectangular platform (30 × 12 × 1 cm) each with a recessed food well (2 cm in depth), over which stimulus objects could be placed. Each platform was positioned near the middle barrier wall, equal distance from the exterior walls of the apparatus, and at a distance of 57 cm from the passageway. The bottom, fifth level contained no platforms or objects and served as the retention interval area during testing. The room contained dim lights (40 lx) and a video camera was positioned in front of the apparatus to record the session for later analysis.

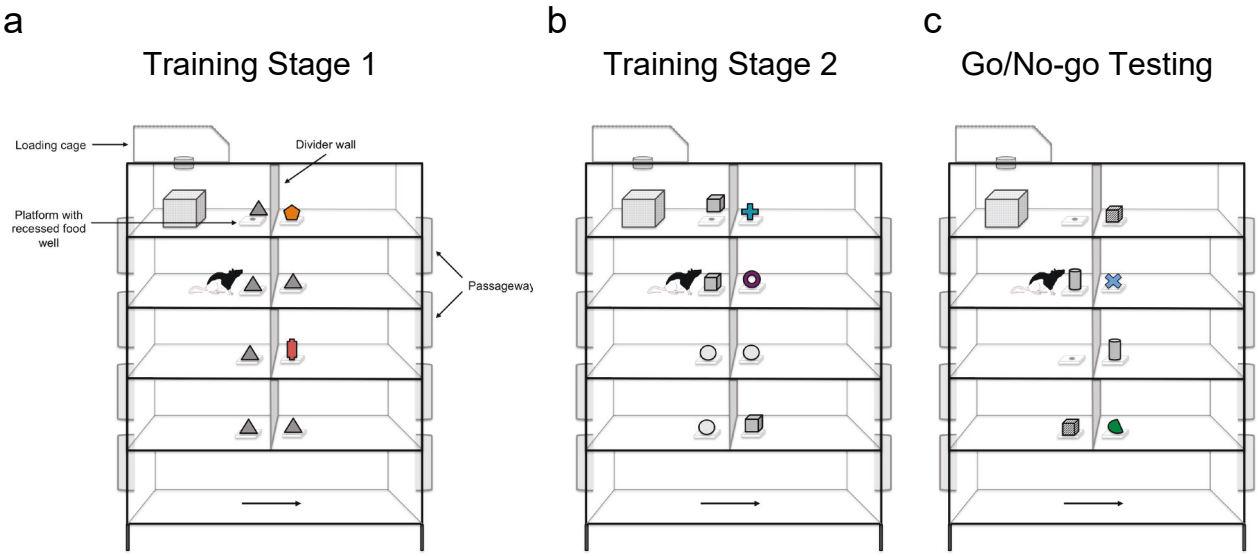

Figure 5.1. Diagrams of the apparatus used for Go/No-go DNMS depicting a session during (a) Training Stage 1 (b) Training Stage 2 and (c) Go/No-go Testing. A loading cage provided access to the apparatus, and passageways on both sides of the apparatus allowed rats to access the different levels. The top four divided levels each contained one plastic platform with recessed food wells in which an object could be placed over. On the sample phase when the rat descended the left side of the apparatus on a training session it encountered either (a) four copies of one sample object on stage 1 or (b) two copies of two distinct sample objects on stage 2. On (c) Go/No-go testing the rat encountered two unique sample objects. For all stages, once the rat reached the bottom level, it traversed to the right side where it ascended each level encountering 4 different test trials. On two trials a copy of the sample object was presented (No-go trial) and on two trials a unique novel object was presented (Go trial).

5.2.2.2. NOP test. The apparatus for the NOP test was an open-field arena (60 × 70 × 70 cm), constructed of grey PVC plastic. The floor of the arena consisted of a stainless-steel tray covered with woodchips. The floor was removable via a slot at the bottom of one wall to facilitate changing the woodchips between each trial. The testing room contained dim lights (14 lx) and a videocamera was positioned over the arena to record the sessions for later analysis.

5.2.2.3. Stimulus objects. A total of 282 different objects were used as stimuli for the Go/No-go task. Objects were made of plastic, metal, glass, or glazed ceramic, and ranged in size from 4 to 18 cm in height, and 4 to 13 cm in width. Each object was large enough to cover the food well but light enough to be easily displaced by a rat. There were at least two copies of each object –one for the learning phase and one for the test. The objects were cleaned using a 70% ethanol solution after every trial on which they were used, as well as at the end of each day.

A separate pool of 10 objects was used for the NOP test. Objects for the NOP test were made of plastic, glass, or glazed ceramic, and ranged in size from 7 to 18 cm in height, and 5 to 13 cm in width. There were at least three copies of each object–two for the familiarization phase and one for the test. A small glass jar (6 cm high) was attached to the bottom of each object with epoxy. The objects were fixed in place by screwing the jars into inverted lids that were attached to the stainless-steel tray in the open field arena (positioned 27 cm from opposing corners). The objects were cleaned after every trial on which they were used, by wiping with a damp paper towel. At the end of each day the NOP objects were cleaned using a 70% ethanol solution.

5.2.3. Procedures

5.2.3.1. Go/No-go DNMS task. The paradigm included a series of training stages. For each stage, rats received one session per day, no fewer than five days per week. There were three stages: 1) habituation and shaping, 2) Go/No-go training, and 3) Go/No-go DNMS.

5.2.3.1.1. Habituation and shaping. On the first seven sessions of habituation all ten rats were placed in the apparatus together for 30 minutes with no stimulus objects present, and ~20 Cheerios (1.8 g, General Mills) were placed on each level to encourage the rats to navigate the environment. The goal of habituation was to have rats complete an entire circuit of the apparatus (start on the top left level and finish on the top right level), with relatively little hesitation. Once the rats were visiting every level within 10 minutes, they were then placed in the environment in pairs (i.e., cage mates) for an average of five sessions. During this stage of habituation and shaping, the rats were introduced to the plastic platforms that contained food wells. One plastic

platform was placed on each level of the apparatus (except the bottom level), and Cheerios were placed inside the food wells. Once a pair of rats was visiting each apparatus level consistently and eating the majority of the Cheerios within 10 minutes, they began to encounter objects. At this point, the rats were placed in the apparatus individually and were gradually familiarized to the procedural aspects of the task, namely to learn to displace objects from over the food wells and to dig for a single Cheerio (~93 mg) buried beneath woodchip (2 cm deep). Burying the Cheerio was done in an attempt to reduce the likelihood that a rat would rely on olfactory cues to locate the reward. Throughout this shaping procedure, the same object, and multiple copies of it, was used. This object was not used in subsequent training or testing. On the first few shaping sessions, the objects only partially covered the food well to encourage timid rats to displace objects. As sessions continued, the objects were gradually positioned to cover the entire food well. On the shaping phase rats required on average 24 sessions (s = 2.75) until they were consistently displacing objects and digging for the Cheerio, at which point they moved onto the training stage.

5.2.3.1.2. Go/No-go training stage 1. The purpose of the training stage was to teach the rats the reward contingency. A session consisted of two phases: a sample phase and a test phase. On the sample phase, the rat descended the left side of the apparatus and encountered four identical copies of one sample object (see Figure 5.1a). One Cheerio was buried beneath each sample object to encourage the rat to approach and investigate it. On the test phase, the rat ascended the right side of the apparatus and received four separate 'trials', one on each level. On two of the trials a copy of the sample object was presented and on the other two trials a novel object was presented. On a trial in which a novel object was presented, two Cheerios were buried beneath it, whereas on a trial with a sample object, no Cheerios were buried beneath it. Accordingly, on the test, the rat learned that encountering a novel object provided a food reward (Go trial), whereas encountering a sample object did not result in reinforcement (No-go trial). The location of the novel and sample objects on the test were counterbalanced across sessions in a pseudorandom order.

The sample and test phase was separated by a short delay in which the rat spent traversing the bottom level of the apparatus. The average time required to traverse the bottom level (the delay) during this stage was 25 s (s = 10.28). Different sample and novel objects were used on each session. On stage 1 a total of 16 different object sets were used, each containing 6

copies of one sample object and 4 unique novel objects. After 16 sessions, rats re-encountered the same object sets in the same sequence starting with the first object set. The first time a rat encountered an object set, the first two novel objects were used on the test, and on the second encounter, the other two novel objects were used.

Between each rat, all of the plastic platforms and objects were cleaned using a 70% ethanol solution. The woodchip on each level was redistributed in an attempt to reduce olfactory cues left by the previous rat.

The dependent measures were *response accuracy* (mean percent correct choices) and *response latency* (mean latency to displace an object). A correct choice was defined as a rat either displacing a novel object from over a food well or not displacing a sample object. An incorrect choice was defined as either a rat displacing a sample object or not displacing a novel object. Latency to displace an object was defined as the time (in seconds) between the moment the rat's four paws touched the test level to the moment the food well was exposed. If a rat did not displace an object from over a food well, it received a latency score of 10 seconds for that trial.[10] Latency to displace an object was scored using ODLog (Macropod software, version 2.6.1).

On the first few sessions, the objects only partially covered the food well to encourage timid rats to displace objects. By approximately the fourth session, the objects were positioned to cover the entire food well. For a particular rat, both response accuracy and latency on the test phase began to be recorded only once all of the objects fully covered the food well.

Two performance criterions were established—one for each dependent measure. The performance criterion that a rat had to reach for response accuracy was at least 80% correct choices on five consecutive sessions (16 correct trials out of 20). Criterion performance for response latency was considered to be attained when the mean latency to displace objects on No-go trials was statistically significantly longer than on Go trials on five consecutive sessions. A rat

[10] The score of 10 s was based on non-displacement times from previous Go/No-go tasks using objects (Cho & Kesner, 1995; Ragozzino, Detrick, & Kesner, 2002). Typically, when a rat does not displace an object the experimenter keeps the rat in the apparatus until the end of the trial, and then it receives the maximum trial time limit as its latency score (e.g., 10 s). In the present experiment, this method was not practical because the rat (not the experimenter) controlled the duration on the trial, and on non-displacement trials the rat tended to quickly leave the test level. This resulted in latencies that were similar to those on Go trials (when a rat would displace the object). Thus, we imposed a set time for non-displacement trials in order to distinguish them from Go trials.

was trained on this stage until it reached both performance criteria or received a maximum of 80 sessions.

5.2.3.1.3. Go/No-go training stage 2. This stage was the same as training stage 1, except now the rat encountered two copies of two different sample objects on the sample phase (see Figure 5.1b). On the test phase, the rat ascended the right side of the apparatus and encountered a third copy of each sample object and two different novel objects. For a particular session, the sample objects on the test phase were presented in the same sequence that the rat had encountered them on the sample phase.

On this stage, eight new object sets were introduced –each containing three copies of two distinct sample objects and four unique novel objects. The new object sets were used in addition to the ones used during stage 1, except now the stage 1 object sets were combined on a session to expose the rat to two distinct sample objects (e.g., on a session a rat encountered half of object set 1 and half of object set 2). A rat was trained on this stage until it reached both performance criteria or until it reached a maximum of 30 sessions. The mean retention interval between the sample and test phase was 25 s (s = 12.32).

5.2.3.1.4. Go/No-go DNMS task. A new pool of 154 objects was used during this stage of testing, and the previous object sets were no longer used. This stage was the same as training stage 2, except now a rat encountered only one copy of two distinct sample objects as it descended the left side of the apparatus (the other two sample levels remained empty). Accordingly, this stage was similar to conventional DNMS tasks in that each sample object was encountered only once during the sample phase. On the test side, as the rat ascended each level it encountered either a copy of a sample object (No-go trial) or a distinct novel object (Go trial) (see Figure 5.1c). Similar to the training stages, two Cheerios were buried beneath each novel object and no cheerios were buried beneath either sample object. On each session, a rat received four trials—two No-go trials and two Go trials. The location of the novel and sample objects on the test phase were counterbalanced in a pseudorandom order. Different objects were used on each session, and the same object served as the sample and novel for each rat. During this stage of testing, the objects were trial-unique such that they never recurred on any session, unlike the objects that were used during training.

A rat was tested until it reached both performance criteria or received a maximum 30 sessions. Rats that failed to reach the response accuracy performance criterion during training

stages were simply tested until they reached the response latency performance criterion.[11] The mean delay between the sample and test phase was 25 s (*s* = 8.23).

5.2.3.1.5. Probe tests. Following Go/No-go DNMS testing, probe tests were administered to confirm the rats were not relying on olfactory cues to correctly locate the food reward buried under the novel object on the test phase. Two types of probe tests were conducted: 1) the food reward was omitted on the Go trials (Go probe) and 2) the sample object was baited on the No-go trials (No-go probe). The probe tests were conducted concurrently with normal tests, such that on one session, two trials were set up as probe tests (one for each respective type) and two trials were normal tests (i.e., novel object baited and sample object not baited). A total of eight probe tests were administered—four of each type. Latency to displace the object was used as the dependent measure and probe test scores were compared to those obtained on the normal tests. The mean delay between the sample and test phase during probe testing was 21 s (*s* = 11.98).

5.2.3.2. NOP test. Rats received NOP testing using a 180-s delay. Prior to NOP testing, rats were habituated to the open field arena for ten minutes daily for two consecutive days. Two identical objects were present in the open field arena during habituation. These objects were not used on subsequent experimental trials. Twenty-four hours following the last habituation session, rats received their first trial. A trial consisted of a familiarization phase and a test phase. For the familiarization phase, a rat was placed in the open field arena and allowed to explore two identical sample objects for five minutes. Following a 180-s retention interval, the rat was returned to the arena which then contained a copy of the sample object and a novel object, and the rat was allowed to investigate for five minutes. Objects were counterbalanced between rats such that the sample objects for approximately half of the rats were used as the novel objects for the remaining rats. The side in which the novel object appeared on was counterbalanced between rats and across trials for an individual rat. Each rat received five trials with a 180-s delay and trials were conducted on different days over a four-week period after rats received Go/No-go DNMS testing. Different object pairs were used for each trial, but the same object pair was used for all rats on corresponding trials. All five object pairs used in this experiment had been previously screened for preference by a different group of rats in a nonchoice test.

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[11] We noticed that with continued testing, some rats had difficulty refraining from displacing objects on the No-go trials, despite exhibiting longer latencies to displace objects on these trials relative to Go trials. Accordingly, we chose to use the performance criterion for response latency rather than response accuracy, as it appeared to be a more sensitive measure of object-recognition abilities. This is further discussed in the Discussion section.

Time spent investigating objects was scored using ODLog (Macropod software, version 2.6.1). The rats were considered to be investigating an object if their head was 4 cm away from the object and oriented towards the object, or away from the object at no more than a 45º angle. A rat standing on its hind legs and touching the object with at least one forepaw was also considered to be investigating. Climbing or sitting on top of an object was not considered investigation. The main dependent measure was the *investigation ratio*. This ratio compares the total object investigation time to the time spent with the novel object during the test phase (Ratio = [$Time_{novel}$/ ($Time_{novel}$ + $Time_{sample}$)]. To determine whether rats' discriminated between the objects, a one-sample *t*-test ($p < .05$) was used to compare mean investigation ratios to chance level of investigation (i.e., a ratio of 0.50). A ratio that was significantly greater than 0.50 indicated the rat spent more time investigating the novel object.

5.2.3.3. Correlational analyses. To determine whether performance on both tasks underlie the same construct, we performed a Pearson correlation comparing scores obtained on the Go/No-go DNMS to those obtained on the NOP test. Presuming an increase in latency to displace objects on No-go trials relative to Go trials reflects a rat's ability to successfully discriminate between familiar and unfamiliar objects, then the latencies can be converted to scores that reflect this ability to discriminate. Accordingly, mean latency scores on the Go/No-go task were converted to Discrimination Ratios [($Latency_{No\text{-}go}$-$Latency_{Go}$)/($Latency_{No\text{-}go}$+$Latency_{Go}$)]. A ratio of zero indicates no difference between the latency to displace sample and novel objects, whereas a ratio above a value of one would signify the rat had a longer latency on No-go trials than on Go trials. We compared the discrimination ratios on the last five sessions of the Go/No-go DNMS to investigation ratio scores on the five NOP tests.

5.2.4. Statistical Analyses

Statistical analyses were performed using the *Statistical Program for the Social Sciences* (SPSS) software for Mac (IBM, version 22). The critical threshold for statistically significant results was set at $p < .05$. Eta-squared and Hedge's *g* are reported as measures of effect size. The 95% confidence intervals (CI) reported are calculated for the respective effect sizes.

5.3. Results

5.3.1. Data Screening

Before conducting any analyses, the data were screened according to the recommended best practices outlined by Kline (2009). The statistical assumptions for one-sample t-tests,

ANOVA, and correlation were verified. All scores were standardized in order to detect the presence of outliers. A z-score greater than 3 was used to describe an outlier (Kline, 2009). Standardized scores for each variable did not reveal the presence of any outliers. Scores on one trial for two rats during the Go/No-go testing phase met the criteria for extreme scores and were excluded from analyses.

The normality of the distribution was assessed for each variable by measuring skewness and kurtosis. Scores were considered normally distributed with a skew less than 3 and a kurtosis less than 10 (Kline, 2009).The distribution of scores was also graphically assessed for normality using a histogram with a normal curve fitted to it. In the current sample, all variables showed acceptable skew and kurtosis, therefore no transformations were applied.

Bivariate scatter plots were investigated to verify the assumptions of linearity and homoscedasticity. A visual inspection of the scatter plots confirmed a linear relationship between variables and confirmed that the homoscedasticity assumption was not violated.

5.3.2. Go/No-go DNMS Task

Scores from the response accuracy were tested for inter-rater reliability. Cohen's κ was computed to assess inter-rater reliability on a random selection of 30% of the trials for each rat. There was excellent agreement between the two raters, $\kappa = .98$, $p < .001$. Moreover, the latency to displace an object on the test trials was tested for inter-rater reliability. Thirty percent of test data were chosen at random for the rater to score. A two-way mixed-effects, absolute-agreement ICC estimate revealed an excellent correlation, ICC = .92, 95% CI [.87, .94], $p < .001$.

5.3.2.1. Training stage 1.

5.3.2.1.1. Response accuracy. Figure 5.2a depicts the mean accuracy scores on the first and last five sessions. The mean score on the first five sessions was 50% ($s = 0$). As training continued, rats obtained a mean score of 80.5% ($s = 1.58\%$) following an average 54 sessions ($s = 13.92$), excluding the criterion sessions. A dependent-samples t-test revealed a statistically significant improvement in scores from the first to the last five sessions of training ($t_{(9)} = -61.00$, $p < .001$, Hedge's $g = -27.3$, 95% CI [-36.26, -18.34]).

5.3.2.1.2. Response latency. Figure 5.2b depicts the mean latencies to displace objects (in seconds) on the first five sessions compared to the five sessions whereby rats had significantly longer latencies on No-go trials compared to Go trials. A dependent samples t-test revealed a statistically significant difference in latencies to displace objects on the first five sessions

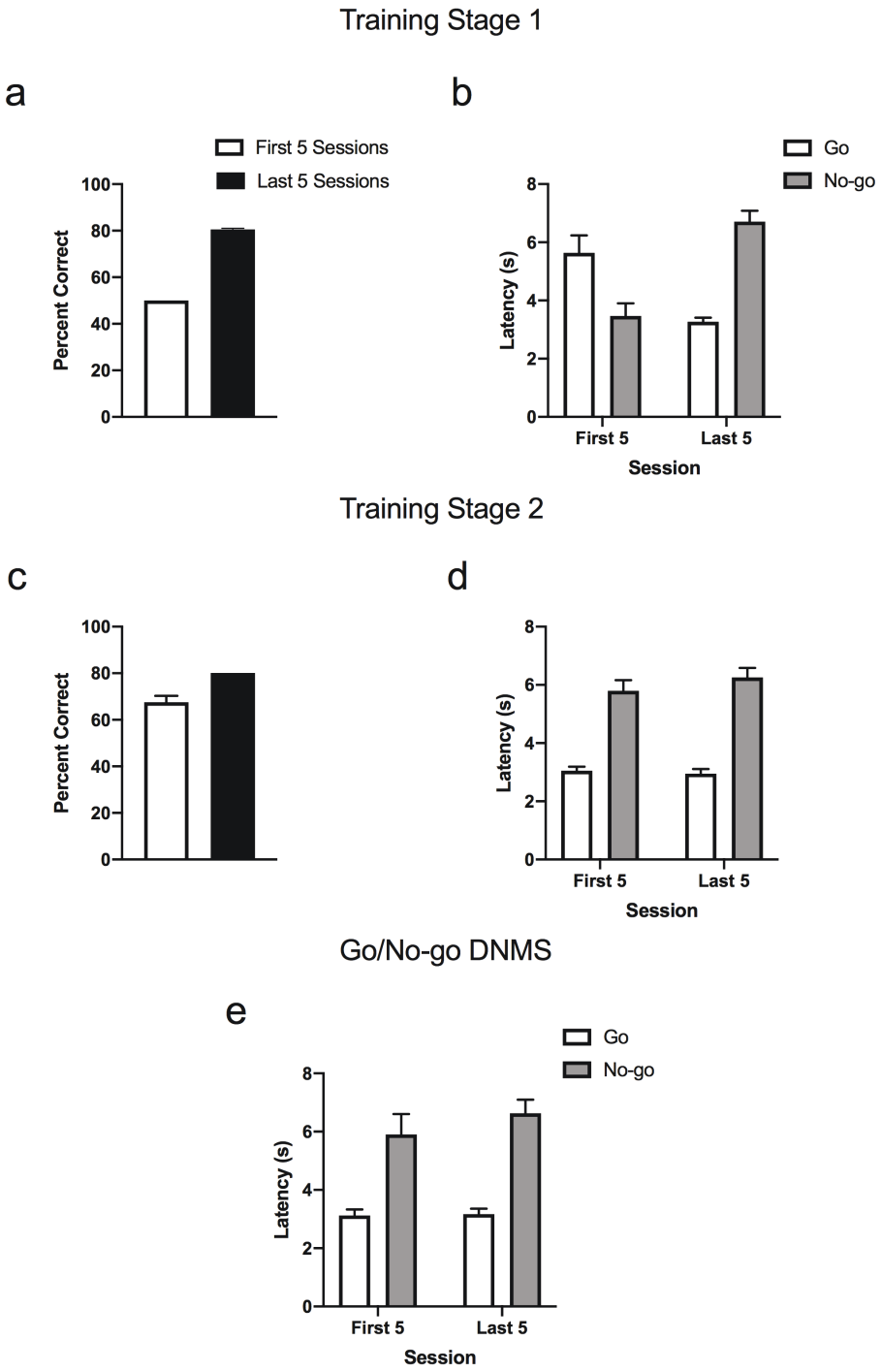

Figure 5.2. Mean accuracy score (± SEM) on the first and last five sessions during (a) training stage 1 and (c) training stage 2. Mean latency to displace objects (± SEM) on Go and No-go trials during the first and last five sessions during (b) training stage 1, (d) training stage 2, and (e) Go/No-go DNMS.

($t_{(9)}$ = -3.34, p = .01, Hedge's g = -1.32, 95% CI [-2.29, -0.34]), with faster latencies to displace objects on No-go trials (M = 3.47, s = 1.36) compared to Go trials (M = 5.64, s = 1.89). This pattern reversed following an average 36 sessions (s = 10.08) (excluding criterion sessions), whereby rats reached five consecutive sessions with significantly longer latencies on No-go trials compared to Go trials ($t_{(9)}$ = 10.81, p < .001, Hedge's g = 3.95, 95% CI [2.39, 5.51]). The mean latency on No-go trials was 6.71 (s = 1.16) and on Go trials was 3.27 (s = 0.44).

5.3.2.2. Training stage 2. One rat became ill during this stage of testing and was euthanized. Thus, the results reported for training stage 2 and subsequent stages are based on nine rats, unless otherwise stated.

5.3.2.2.1. Response accuracy. Three of the nine rats failed to reach the performance criterion within the allotted 30 sessions. Accordingly, data for all three rats were excluded from the statistical analysis. The mean accuracy score during the first five sessions was statistically significantly above chance (M = 67.5%, s = 6.89%). Rats reached a mean score of 80% (s = 0%) following an average 5.17 sessions (s = 2.4), excluding the criterion sessions (see Figure 5.2c). A dependent-samples t-test revealed a statistically significant improvement in scores from the first to the last five sessions of training ($t_{(5)}$ = -4.44, p = .01, Hedge's g = -2.56, 95% CI [1.28, 3.85]). The highest level of accuracy for the rats that failed to reach the performance criterion was 75%, which was reached by Session 15 for one rat and Session 18 for the remaining two rats.

5.3.2.2.2. Response latency. The data for all nine rats were included in the analysis on latency to displace objects (Figure 5.2d). A dependent samples t-test conducted on the first five sessions revealed that rats maintained a tendency to take significantly longer to displace objects on No-go trials (M = 5.80, s = 1.09) compared to Go trials (M = 3.05, s = 0.43) ($t_{(8)}$ = 8.32, p < .001, Hedge's g = 3.32, 95% CI [1.84, 4.79]). As testing continued, all nine rats exhibited statistically significantly longer latencies to displace objects on No-go trials compared to Go trials on five consecutive sessions ($t_{(8)}$ = 11.55, p < .001, Hedge's g = 4.34, 95% CI [2.58, 6.11]). Notably, this was achieved following an average 1.11 sessions (s = 1.96). The mean latency on No-go trials was 6.26 (s = 0.97) and on Go trials it was 2.95 (s = 0.47).

5.3.2.3. Go/No-go DNMS.

5.3.2.3.1. Response accuracy. The majority of rats had difficulty refraining from displacing the sample object on No-go trials. Only three of the nine rats reached the performance criterion within 30 sessions. The three rats reached a mean accuracy of 81.67% (s = 2.89%)

following an average 5 sessions (s = 4.36). The six rats that failed to reach criterion only withheld responding on an average of 16% of the No-go trials (s = 11%; min = 0% and max. 32%). The highest level of accuracy for rats that failed to reach the performance criterion was 75% for two rats, which was reached by Session 5 and 21, and 65% for three rats which was reached by Session 6, 8, and 18. Lastly, one rat never withheld responding and displaced the object on every No-go trial.

5.3.2.3.2. Response latency. All nine rats successfully reached five consecutive sessions whereby they had mean latencies that were significantly longer on No-go trials compared to Go trials (see Figure 5.2e). This was achieved by an average 2 sessions (s = 3.12). A dependent samples t-test conducted on the last five sessions revealed a statistically significant difference in latencies to displace objects ($t_{(8)}$ = 8.41, p < .001, Hedge's g = 3.71, 95% CI [2.13, 5.29]), with longer latencies to displace objects on No-go trials (M = 6.76, s = 1.39) compared to Go trials (M = 2.97, s = 0.37).

5.3.2.4. Probe tests. Mean latency to displace objects during probe testing is plotted in Figure 5.3. A two-way repeated measures ANOVA revealed a significant main effect of Test Condition ($F_{(1,8)}$ = 10.21, p = .01, partial η^2 = .56), a significant main effect of Object Type ($F_{(1,8)}$ = 9.5, p = .02, partial η^2 = .54), and no significant interaction ($F_{(1,8)}$ = 0.79, p = .40, partial η^2 = .09). A follow-up t-test (Bonferroni-corrected) revealed that the latency to displace sample objects was statistically longer compared to novel objects (p = .01).

5.3.3. NOP Tests

Rats spent on average 57.63 s (s = 11.77) investigating objects during the 5-min familiarization phase. On the test phase, an average investigation ratio was calculated for each rat, based on the five NOP trials using the first two minutes of the test. Trials were excluded if a rat spent less than one second exploring either object on the test. This resulted in the exclusion of one trial for two rats. One-sample t-tests (one-tailed) revealed that mean investigation ratios were statistically significantly above chance level ($t_{(8)}$ = 3.50, p < .01, Hedge's g = 1.77, 95% CI [0.51, 3.03]). Scores remained statistically significantly above chance for all cumulative minute bins of the test phase. The time spent investigating objects on the test trials were tested for inter-rater reliability. Thirty percent of test data were chosen at random for the rater to score. A two-way mixed-effects, absolute-agreement ICC estimate revealed an excellent correlation, ICC = .99, 95% CI [.98, .99], p < .001.

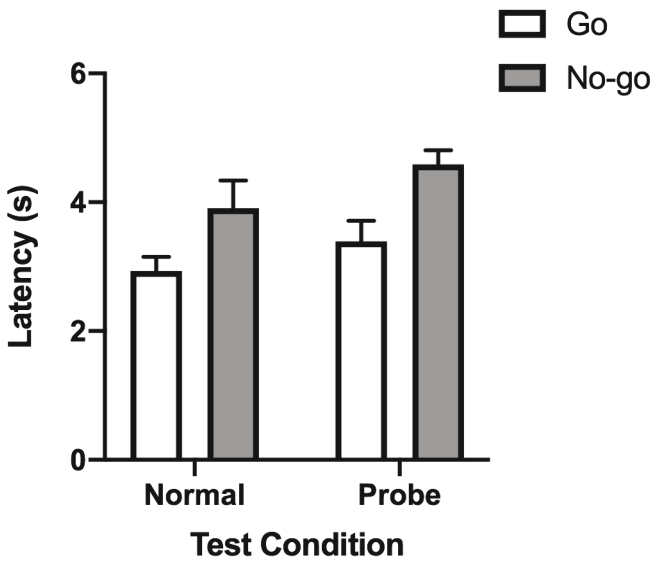

Figure 5.3. Mean latency (± SEM) to displace objects on Go and No-go trials on probe and normal tests.

5.3.3.1. Correlations. Pearson correlations were conducted on the total time investigating sample objects during the familiarization phase and subsequent mean investigation ratios. There was no significant correlation (r = .24, p = .54, 95% CI [-.50, .78]).

5.3.4. Correlation between scores on the Go/No-go DNMS and NOP test

Figure 5.4 shows the results of a correlational analysis of Go/No-go DNMS discrimination ratios and NOP investigation ratios. There was no significant correlation between average Go/No-go DNMS discrimination ratios and average NOP investigation ratios (r = -.08, p = .83, 95% CI [-.61, .70]), indicating that performance on the Go/No-go DNMS was not significantly correlated with the magnitude of novelty preference on the NOP test.

5.4. Discussion

During training stage 1 all rats reached the performance criterion of 80% correct choices on five consecutive sessions following an average 54 sessions (excluding criterion sessions). In terms of latency to displace objects, all rats exhibited significantly longer mean latencies on No-go trials compared to Go trials following an average 36 sessions. Both results suggest that rats acquired the reward contingency, and successfully discriminated between familiar and unfamiliar objects on the test. As the demands of the task were made more difficult with the introduction of an additional sample object during the sample phase, rats maintained significantly longer latencies to displace objects on No-go trials compared to Go trials. We noticed, however, that with continued testing, the majority of rats failed to refrain from displacing objects on No-go trials. This finding was unexpected, as we had predicted that rats would be less likely to displace a sample object on the test after increasing the cost for making an error. The latter finding suggests that in the present paradigm, using *latency to displace objects* on the test provides a more sensitive estimate of object-recognition memory compared to using *percent correct choices*. Moreover, compared to response accuracy, latency to displace objects remained stable over continued testing, as evidenced by the significant difference in latencies within the first few sessions on training stage 2 and the Go/No-go DNMS stage.

We can be confident that the rats were not relying on olfactory cues to successfully locate the food reward, as the results from the probe tests revealed no significant difference between latencies to displace objects on the probe and normal tests. Thus, rats still exhibited significantly longer latencies to displace sample objects compared to novel objects on both types of probe tests.

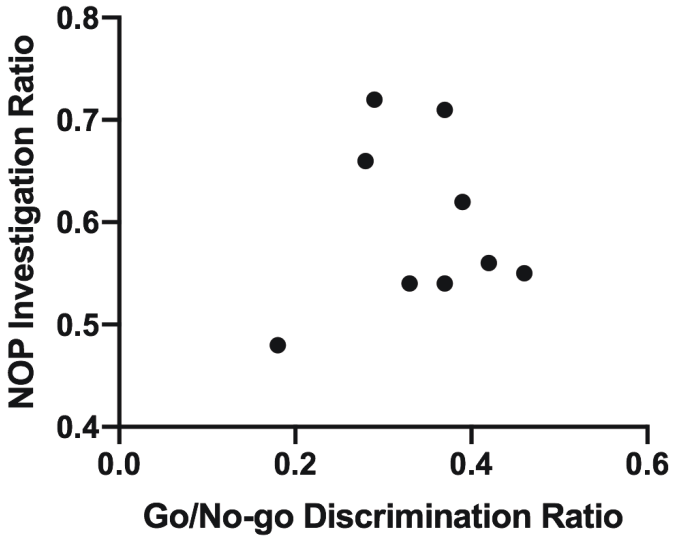

Figure 5.4. Scatterplot of scores of individual rats obtained on the Go/No-go DNMS task and on the NOP test. Scores on the two tests were not significantly correlated.

As training continued during stage 2, it became clear that rats had difficulty inhibiting their response to displace the sample object once in close proximity to it. Consequently, if we only relied on mean percent correct choices as an index of object-recognition memory, we would have recorded these responses as errors and concluded that the majority of the rats failed to either acquire the reward contingency or discriminate between familiar and unfamiliar objects on the test. However, measuring their *latency to displace* an object made it clear that the rats had learned the reward contingency and successfully discriminated between objects on the test as exhibited by their longer latencies on No-go trials compared to Go trials. This finding highlights the importance of developing a behavioural paradigm that can accurately measure the construct under investigation and incorporates appropriate operational definitions for the variable of interest. Indeed, including multiple dependent measures to assess memory has been shown to be useful on other rodent behavioural tests. For example, on radial arm maze tasks designed to assess spatial memory, a rat collects a food reward that is located at the end of several extended arms. A correct choice is typically defined as the rat entering a maze arm one time (one baited with food), and an error is defined as the rat re-entering a previously visited arm (one that is no longer baited with food). In one study, researchers decided to also include the latency to reach the end of the maze arm as one of the dependent variables. On trials in which rats made an "error" the researchers found that rats exhibited significantly longer latencies to reach the end of the arm compared to the first visit (Brown & Cook, 1986). This finding suggested that rats remembered the previously visited arm, and that relying solely on choice accuracy did not provide the most accurate estimate of recognition memory (Brown & Cook, 1986). Accordingly, relying on multiple behavioural measures is useful because it can reveal information about the status of memory that may otherwise go undetected.

It is not necessarily possible to eliminate all potential confounds that may interfere with accurate measures of a variable, but through careful observation of behaviour one can become cognizant of potential confounds and attempt to reduce them accordingly. Previous research has shown that using "first choice" as a measure of accurate memory on a discrimination test does not necessarily reflect the true status of memory (Carr & Wilkie, 1997; Deibel & Thorpe, 2013; Means, Pia, Ginn, Pence, & Watson, 2000; Mistlberger, De Groot, Bossert, & Marchant, 1996; Wilkie, Willson, & Carr, 1999). Put differently, "errors" on a task do not necessarily reflect a failure in memory. In a time-place discrimination task, rats were trained to retrieve a food reward

by pressing one lever during a morning session and a different lever during an afternoon session. The researchers measured the first lever press at the start of each session as a means to determine whether rats could use time-of-day cues to correctly locate the food reward. They found that at the start of each session the rats pressed equally on both levers, which the researchers initially took to indicate that the rats failed to discriminate which lever provided food at which time of day (Wilkie et al., 1999). However, when the researchers provided rats with a brief period to 'patrol' the apparatus prior to the start of each session, their lever response accuracies on the test were significantly above chance. This finding revealed that the rats could, in fact, accurately discriminate which lever provided food at each respective time of day. The tendency for the rats to randomly press levers at the beginning of a session appeared to be a byproduct of exploring the environment, and not a reflection of their memory for time of day information (Wilkie et al., 1999). Thus, by making careful observations it can reveal potential confounds in a task design, which can allow for modifications to the procedure to provide a more straightforward interpretation of the behaviour as it relates to the construct under investigation.

Rats displaced the sample objects on the test despite recognizing them and despite having learned that displacing those objects would not be rewarded. This was evidenced by their longer latencies to displace sample objects than novel objects associated with food reward. Moreover, we observed that rats would sometimes displace an object on a No-go trial and not dig in the food well. This suggests that upon displacing the object the rat did not expect to find a food reward. Thus, in the present paradigm 'displacing an object' does not appear to provide an accurate reflection of a rat's ability to discriminate the familiarity of a previously encountered object. The tendency to displace sample objects on the test may reflect an exploration of the features in the environment. The findings from this experiment, in addition to previous research revealing that 'first choice' may not accurately reflect the status of memory, raises questions regarding the observed accuracy scores we previously reported using the mDNMS task (Cole et al., 2019). To what extent was displacing a sample object first on the test a reflection of the rat's memory for the sample object and to what extent was it exploration of the environment? Unfortunately, based on the available data, this question cannot be answered. However, what is clear based on the present findings is the importance of modifying a procedure to eliminate potential confounds and including multiple behavioural measures to study an underlying construct.

The lack of a significant correlation between scores on both tasks, suggest the two tasks are not measuring the same underlying cognitive processes, one of which is object-recognition memory. The latency scores on the Go/No-go DNMS were converted to discrimination ratios in order to provide an indirect measure of the associative strength of memory for the sample object. There are several reasons why we are confident that the Go/No-go DNMS provides a measure of a rat's object-recognition memory. First, by requiring the rat to make an instrumental response with which it makes an explicit choice between familiar and unfamiliar objects, and by providing a reward for accurate responses, it teaches the rat the successful strategy it must employ to reach the goal. Consequently, this increases the likelihood that the behaviour on the test reflects what you think it does, which is object-recognition memory. Secondly, by teaching the rat the successful strategy to employ, it reduces the number of alternative explanations for the observed behaviour on the test, which allows for a more straightforward interpretation of the observed behaviour. Thirdly, by collecting several trials within a session for each individual, it provides a reliable indication of their ability to discriminate between a familiar and unfamiliar object. Lastly, by conducting probe tests, we ruled out the possibility that rats were relying on olfactory cues to locate the food reward. Thus, there is no other plausible means for why a rat would exhibit longer latencies on No-go trials compared to Go trials, other than the fact that the rat must recognize the sample object. Altogether, the discrimination ratios can be confidently taken as an index of a rat's memory for the sample object. Conversely, on the NOP test, interpreting what the magnitude of a novel-object preference signifies in relation to the persistence or accuracy of the rat's memory for the sample object is not as straightforward, especially when a rat fails to exhibit a novelty preference. On the NOP test, there are several reasons why a rat might not exhibit a novelty preference. The rat may not recognize the sample object, or it does recognize the sample, but does not prefer to explore the novel object. A rat's spontaneous investigation of a novel object can be modulated by internal attributes, such as stress levels, whereby higher stress levels reduce the tendency to approach novel objects (Gulinello et al., 2018; Hughes, 1997), or by external attributes related to test conditions (Berlyne, 1955; Besheer & Bevins, 2000; Blaser & Heyser, 2015; Ennaceur, 2010; Wilkinson, Herrman, Palmatier, & Bevins, 2006). For example, object attributes such as size, texture, and complexity can induce different amounts of investigation (Berlyne, 1955; Chemero & Heyser, 2005; Ennaceur, 2010; Heyser & Chemero, 2012). Objects that can be climbed over and ones that have complex

features elicit greater levels of exploration that do not decline with successive presentations compared to objects that cannot be climbed on or have simple features (Berlyne, 1955; Chemero & Heyser, 2005; Heyser & Chemero, 2012). Moreover, increasing the amount of exposure to the testing environment prior to the familiarization phase increases sample object investigation during the familiarization phase. The latter is thought to occur because other competing behaviours, such as exploring features of the apparatus, are reduced (Besheer & Bevins, 2000; Sheldon, 1969). These findings reveal the delicate nature of the testing conditions when using the NOP test and the number of potential factors that can influence behaviour on the test. Accordingly, this demonstrates how it can be difficult to conclude with certainty that the preference score reflects the extent to which the rat recognizes the sample object, and not some alternative factor influencing behaviour on the test. Moreover, given the number of variables that can influence a rat's preference to explore objects on the test, when a rat fails to show a novelty preference on the test, it is difficult to determine what this means in relation to the status of object-recognition memory. The number of reasons why a rat fails to exhibit a novel-object preference on the test is complicated by the fact that the NOP test does not involve a goal, and thus rats are not required to make an explicit choice response based on their memory for the sample object. On the basis of this task analysis, one can infer that the Go/No-go DNMS is providing a measure of object-recognition, whereas behaviour on the NOP test may reflect a number of alternative explanations besides object-recognition memory. Accordingly, the lack of a significant correlation between scores on the two tasks suggests that the magnitude of a rat's preference to explore novel objects is not related to its ability to discriminate between familiar and unfamiliar objects on the Go/No-go DNMS task.

It is important to note that the lack of correlation between scores on both tasks may reflect differences between the procedures used for each task. For example, the delay used on both tasks was different (e.g., 25 vs. 180 s). Thus, it could be argued that the memory demands on the NOP test were more taxing than on the Go/No-go DNMS task, which could explain differences in the scores on both tasks. Moreover, there were differences in the task characteristics, which have been previously hypothesized to affect correlational analyses. The authors of a previous study failed to find a strong correlation between measures of both working- and reference-memory when comparing spatial memory performance using the radial arm maze and holeboard (van Luijtelaar, van der Staay, & Kerbusch, 1989). The authors posited that the

low correlation between scores on each task might reflect differences in terms of the spatial cues that the rats relied on (intra- vs. extra-maze cues) and the operational definition for a "choice," specifically the ease with which to make a choice (e.g., running down a maze arm vs. simply poking the nose into a hole in the floor). Thus, although both tasks are said to provide measures of spatial working- and reference-memory, they fail to correlate due to differences in the demands of the task. In the present experiment, the cues that a rat could rely on to successfully perform both tasks was the same: the features of the objects. On the Go/No-go DNMS task you can be confident that performance on the test reflects the ability to discriminate the familiarity of a previously encountered object. Rats are trained to learn that the features of the object are integral for successful performance on the task (i.e., retrieving a food reward). Presumably, then on the sample phase the rat should focus more on actively learning (encoding) the features of the sample object in order to successfully select a future response to retrieve the food reward. Conversely, on the NOP test, the rat is simply placed into an arena that contains two objects and is provided an opportunity to explore the environment. Given this is a test of incidental learning, which lacks an unambiguous instrumental behaviour, other factors can guide behaviour on the test that may not relate simply to the rats ability to discriminate between familiar and unfamiliar objects (as described above). Moreover, on the familiarization phase the rat is presumably encoding information not only about the sample objects but also their location relative to each other and their surrounding context. Consequently, on the test a rat might rely on spatial and nonspatial information to successfully discriminate between the sample and novel object. Thus, compared to the Go/No-go DNMS task, on the NOP test there are more potential variables influencing behaviour on the test, besides object-recognition memory.

Additionally, in the present experiment, the operational definition used for object-recognition on both tasks was quite different, which can explain the lack of correlation. Indeed, a major threat to construct validity—whether scores on a task reflect the specific construct it was designed to measure—is when the operational definition is confounded by other constructs (Kline, 2009). That is why it is important to design a task whereby alternative explanations for the observed behaviour can be easily ruled out. In terms of the NOP test, relying on the *amount of time spent investigating* the novel object relative to the sample as an estimate of the persistence or accuracy of memory for the sample likely reflects other behavioural responses besides object-recognition memory (as described above). This is a drawback of the NOP test, as

one cannot tease apart the extent to which the behavioural response reflects object-recognition abilities and some alternative factor. Thus, it is likely that behaviour on the NOP test does not necessarily reflect only object-recognition memory. Conversely, on the Go/No-go DNMS task, using *latency to displace an object* provides a more sound measure. By training the rat to make an explicit choice response between familiar and unfamiliar objects and by rewarding accurate choices, it reduces the number of these unsolicited behavioural responses that may obscure the expression of object-recognition memory. Consequently, the observed behaviour on the test is more likely to reflect what the task was designed to measure—object-recognition memory.

The present findings revealed that performance on the Go/No-go DNMS task remained consistent over time. An important next step would be to determine whether the Go/No-go DNMS can be used to assess memory for objects spanning longer periods of time (e.g., several minutes or hours). The Go/No-go DNMS task consists of several advantageous features, namely: 1) the involvement of an instrumental response with which the rat makes an explicit choice between familiar and unfamiliar objects, 2) a reward for accurate choices, and 3) the possibility of testing individual rats on several trials per session. Moreover, unlike the existing DNMS tasks, the Go/No-go DNMS eliminates the potential for rats to develop positional biases and allows for the inclusion of multiple dependent measures to assess object-recognition memory. Collectively, these features provide a less ambiguous interpretation of behaviour in relation to object-recognition memory compared to the NOP test.

Chapter 6: General Discussion

The primary goal of the experiments in this thesis was to develop a new and improved object-recognition memory task for rats—one that addressed the known limitations of conventional DNMS tasks and the NOP test. The drawbacks associated with the existing DNMS tasks are that they require extensively training rats, are difficult for inexperienced experimenters to effectively employ, and rats' accuracy scores decline when tested on delays lasting more than a few minutes. Consequently, conventional DNMS tasks are labour intensive and cannot be used to assess long-term object-recognition memory in rats. The limitations of the NOP test are that it is not possible to discern what a lack of novelty preference on the test signifies in relation to memory, and it is not clear to what extent the magnitude of novelty preference on the test reflects the strength in memory for the sample object. Accordingly, the NOP test does not provide a straightforward estimate of a rat's object-recognition abilities. To address the drawbacks of the existing tasks, there were four objectives in mind when we created the mDNMS task, namely we wanted a task that: 1) rats could master quicker than conventional DNMS tasks, 2) was simple for the experimenter to employ, 3) could be used to assess long-term object-recognition memory, and 4) provided a straightforward interpretation of behaviour as it relates to object-recognition memory.

The following section (Section 6.1) provides a summary of whether or not the objectives of this goal were achieved. Next, a discussion on the refinements made to the paradigm is provided (Section 6.2). A secondary goal of this thesis was to compare performance on the mDNMS task to that of the NOP test as a means to validate the latter. Section 6.3 includes a summary of the findings in relation to this objective. Thereafter a discussion on the divergent findings between performance on the NOP test and mDNMS task is provided (Section 6.4), followed by a discussion on the theoretical implications of the findings (Section 6.5). Next, the limitations of the mDNMS and Go/No-go DNMS task are addressed (Section 6.6) followed by future directions (Section 6.7) and conclusions (6.8).

6. 1. Does the mDNMS task address the drawbacks associated with existing tasks?

The following section summarizes whether or not the mDNMS task addresses the drawbacks of the existing tasks and successfully meets the objectives that were outlined at the beginning of the thesis.

6.1.1. Addressing objective #1: Extent of required training

Across the studies that have used the DNMS procedure developed by Mumby and colleagues, the average number of trials rats required to reach the performance criterion (34 correct choices out of 40) ranged between 174-420 trials. By comparison, rats in the experiment described in Chapter 2 required an average of 24 trials to reach the criterion of at least 16 correct choices out of 20 on five consecutive sessions. Comparatively, rats in the experiments described in Chapter 3 and Chapter 4 (Sham-operated rats) required more trials to reach criterion (an average of 79 trials). Nevertheless, they required significantly fewer trials compared to previous DNMS studies. Notably, the mDNMS task acquisition rate was faster compared to previous studies despite using a longer retention delay and presenting four distinct sample objects compared to one sample object. Thus, rats retained more item information over a longer delay compared to rats in previous studies and were capable of reaching comparable choice accuracy levels in significantly fewer trials. It would appear that rats' performance on the mDNMS task is more robust compared to conventional DNMS tasks.

Several factors are likely to have contributed to making the new mDNMS task relatively easy for rats to learn and perform, and for experimenters to administer in a consistent manner. On conventional DNMS procedures developed in the 1980s, the experimenter plays an interactive role in administering individual trials. Rats probably perceive humans as large, noisy, smelly potential predators, and a rat that perceives the experimenter as the most interesting thing in the room will pay more attention to the experimenter than to the task at hand. A rat, especially a timid one, can startle easily if the experimenter makes sudden, quick movements or noises (i.e., sign of a potential threat, such as a predator). The mDNMS task apparatus has the advantage that the objects can be set up before each trial, and after the rat is placed in the loading cage, the experimenter can quietly leave the room, allowing the rat to "self-administer" trials. Eliminating the presence of the experimenter lessens the potential for distraction.

The pre-training procedure also likely contributed to the above-chance level of initial performance. On conventional DNMS tasks, pre-training typically consists of administering object-discrimination problems, which entails repeatedly presenting the same two distinct objects to the rat where selection of one of the objects is rewarded and selection of the other is not (Kesner et al., 1993; Mumby et al., 1990). This teaches the rat both the instrumental-response requirements of the task (i.e., displace objects for food) and that the visual/tactile object features

are key to predicting food location. The pre-training procedure in the present study incorporated these task characteristics in addition to teaching the rat that displacing the sample object on the test phase would not provide a reward. Presenting multiple copies of the same sample object within sessions increased the opportunity of the rat to learn this feature, which may have further facilitated task acquisition

The pre-training procedure varied considerably between the mDNMS task and conventional DNMS. By excluding the number of pre-training sessions in the calculation for the number of trials to reach criterion, it could be argued that it obscures the findings. Accordingly, including the pre-training sessions in the calculation for the average number of trials needed to master the task, it was revealed that rats required on average 178 trials in the experiments described in Chapter 2, 3, and 4, respectively. Comparatively, the average number of pre-training and training trials rats required on the DNMS task developed by Mumby et al. is ~400 trials (see Table in Appendix A). Thus, in line with our objective, these findings confirm that the new mDNMS task can be mastered in considerably fewer trials compared to conventional DNMS tasks.

6.1.2. Addressing objective #2: Simple for the experimenter to employ

Given the active role that the experimenter plays in conventional DNMS tasks, the experimenter must be trained on how to effectively administer trials without influencing the rat's behaviour on the test. The most difficult aspect of DNMS testing for someone inexperienced with the behaviour of laboratory rats is learning how not to distract the rat with certain movements and sounds while administering the test (as described above). The mDNMS task apparatus has the advantage that the objects can be set up before each trial, and after the rat is placed in the loading cage, the experimenter can quietly leave the room. This aspect also reduces the potential for experimenter bias, such as the experimenter unintentionally giving cues to the rat as to which object will be rewarded on the choice test. Accordingly, compared to conventional DNMS tasks, it is much easier and faster to train several experimenters on how to effectively administer a test session on the mDNMS task. Moreover, on conventional DNMS tasks the experimenter stands for hours each day administering trials—a feature that most individuals who have used it would agree is rather unpleasant. Conversely, when using the mDNMS task, the experimenter has the opportunity to sit and observe the rat on a TV monitor in an adjacent room. Thus, compared to conventional DNMS tasks, the mDNMS procedure is less

labour intensive and less complicated to effectively employ for someone who is inexperienced with the behaviour of laboratory rats.

6.1.3. Addressing objective #3: Testing long-term memory

The existing DNMS paradigms cannot be used to study long-term memory for objects because rats cannot perform accurately once the delay exceeds more than a few minutes. Rats in the experiment described in Chapter 2 maintained good performance as the retention interval between the sample and test phase was increased. Accuracy scores declined as the retention interval was increased to 630 s but they remained significantly above chance. These levels of asymptotic performance compare favorably with the asymptotic levels observed at similar retention delays on conventional DNMS tasks (see the Table in Appendix A). Performance following the 4 hr delay, however, was poor. Accordingly, by modifying the sample phase procedure such that rats received several distributed sample object exposures, we observed that rats could successfully discriminate between familiar and unfamiliar objects on the mDNMS task following delays lasting 72 hr, 3 weeks, and ~45 weeks (Chapter 3). Accordingly, we demonstrated that modifying the mDNMS sample phase procedure allowed for assessing rats' memory for objects following long retention intervals.

6.1.4. Addressing objective #4: Straightforward interpretation of behaviour

There were two aspects of the mDNMS task that can be analyzed to determine whether or not it provides a clear estimate of object-recognition memory: 1) the extent to which the observed behaviour on the test reflects the construct under investigation and 2) the ease with which to rule out interpretational ambiguity. These two aspects are discussed below.

6.1.4.1. mDNMS scores reflect object-recognition abilities. The mDNMS task has good *face validity* in terms of the theoretical definition of object-recognition memory—the ability to discriminate the familiarity of a previously encountered object. Subjectively, the task meets this goal as it entails presenting a rat with a sample object to learn, followed by a test whereby the rat makes a choice between displacing either the sample or a novel object. By training the rat to learn that it will receive a food reward when it displaces the unfamiliar object on the test, it provides the opportunity to instruct the rat on the purpose of the task. When a rat exhibits a tendency to displace unfamiliar objects over familiar objects on the test to a high criterion, then you can be more certain that it is engaging in this behaviour because it has learned the reward contingency and is applying it. Consequently, the observed behaviour on the test can more

confidently be taken to reflect the rat's ability to discriminate between familiar and unfamiliar objects.

The operational definition of object-recognition memory on the mDNMS task—percent correct choices on the test—adds to its construct validity. Compared to studying memory in human adults, studying memory in nonhuman animals is more difficult because you can only rely on observable (nonverbal) behaviour. The subject must produce a response that indicates with confidence *which* stimulus was previously encountered and which stimulus was not. This feat is made far easier by training the subject (giving instructions) to produce an explicit choice response with which one can infer that the behaviour reflects memory for the previously encountered object. By reinforcing the rat's behaviour to displace an unfamiliar object when it is presented next to a familiar one (or when presented alone, as in the case with the Go/No-go DNMS) and by setting a strict performance criterion whereby the rat is required to reach a high level accuracy, it increases the confidence that when this behaviour is observed on the test it reflects the rat's ability to recognize the sample object. Additionally, by collecting many of these discrete tests from each individual rat across sessions, it provides more certainty that the observed behaviour is a true reflection of the rat's underlying object-recognition abilities, as it reveals the consistency of the behaviour while maximizing the sensitivity of the measure. Intuitively, across 100 trials, if one rat gets 90% correct and another rat gets 65% correct, it seems easier to accept that the first rat has better recognition than the second rat. Conversely, on the NOP test when comparing a 90% novelty preference ratio in one rat to a 65% novelty preference ratio in another rat, it is not as easy to make the same assumption. Accordingly, a rat's performance on the mDNMS task, compared to the NOP test, can more confidently be interpreted as reflecting object-recognition abilities because it incorporates: 1) an unambiguous instrumental response with which the rat makes an explicit choice between familiar and unfamiliar objects, 2) a reward for accurate choices, and 3) the possibility of testing individual rats on several trials per session, with each trial consisting of an independent test of recognition memory. Also, under these conditions it helps narrow down the number of alternative explanations for the observed behaviour on the test compared to the NOP test where no instructions are provided. Altogether, this increases the level of confidence one has in the interpretation of the behaviour and increases the likelihood that the behaviour provides an accurate estimate of the internal construct.

The significant improvement in scores on the first five sessions compared to the final five criterion sessions during mDNMS task acquisition in each experiment presented in this thesis indicates with confidence that the rats acquired the nonmatching rule and revealed that they successfully discriminated between familiar and unfamiliar objects. Moreover, rats' scores on the mDNMS task following the 180-s delay were reliable, indicating that their ability to discriminate between familiar and unfamiliar objects on the test remained consistent over time (Chapter 2). A key feature when determining the construct validity of a task is that the scores are reliable. Moreover, the overall results from the probe tests allow us to confidently rule out the possibility that rats successfully solved the task by relying on olfactory cues.

Lastly, after refining the mDNMS task to include a Go/No-go procedure, rats exhibited significantly longer latencies to displace sample objects compared to novel objects, indicating that they acquired the reward contingency and successfully discriminated between the familiar and unfamiliar objects on the test. Moreover, on the Go/No-go DNMS task, using latency to displace an object from over a food well, compared to choice accuracy (displacing only the novel object), provided a more sensitive measure of object-recognition memory.

6.1.4.2. Ruling out interpretational ambiguity. On the NOP test, it is difficult (perhaps impossible) to determine what a lack of novelty preference signifies in relation to memory. An essential aim when developing a behavioural task is the ease with which to rule out alternative explanations when interpreting behaviour. Like any behavioural task, there can be challenges when interpreting the basis for performance deficits on a DNMS task. Performance deficits can reflect an object-recognition impairment or some alternative factor. For example, one concern that has been raised when using DNMS tasks surrounds potential confounds that are introduced due to the appetively-motivating aspect of the task (Ameen-Ali, Easton, & Eacott, 2015; Ennaceur, 2010; Ennaceur & Delacour, 1988). When a rat receives a treatment and is subsequently impaired on the task, it has been argued that it is difficult to determine whether the treatment has disrupted the memory for the objects or the motivation for the food reward. Although a treatment can affect a rat's motivation for food, there are certain observations that can be made to exclude motivational factors as a potential confound. For example, measuring the latency to displace objects and the speed at which the food reward is consumed in both the control and treatment group can help rule out potential motivational differences. If there are no significant differences on these measures between the control and treatment group, then it is

reasonable to presume the treatment has not affected a rat's motivation for the food reward. Accordingly, being able to rule out alternative explanations for the observed behaviour on the test is an advantage of the mDNMS task over the NOP test.

Another concern with using appetitively-motivated tasks is the tendency to restrict rats' daily food intake to maintain them at a body weight of 85-90% of the weight of matched free-feeding rats. This amount of food restriction can make rats hyperactive, which can produce hasty responding that interferes with accuracy on the test. This, however, need not be a concern because typically rats only require food restriction during preliminary stages of training when they are acquiring the procedural aspects of the task. After training, the amount of daily food can be increased such that the rats are maintained at a body weight of 95-100% of the weight of free-feeding rats. Including these design measures during testing can help rule out other potential reasons for performance deficits.

Another feature of DNMS tasks is that rats must first learn a response reward association (i.e., the nonmatching rule) and apply it on every trial. When a rat exhibits a DNMS deficit on the test after receiving some form of treatment, it may be unclear as to whether the treatment has produced an object-recognition impairment or disrupted the ability to acquire or apply the reward contingency. One way to avoid the problem of rats failing to acquire the rule is to provide extensive training on the procedural aspects of the task *prior* to administering a treatment. Following treatment, if the rat readily approaches objects, displaces them, and consumes the reward then it clearly remembers the procedural aspects of the task. To confirm that a treatment did not disrupt the ability to apply the reward contingency, a comparison can be made between the control and treatment group's performance following both short and long retention intervals. If accuracy on the task for both groups is not significantly different following the shortest retention interval (when memory load demands are low), then it is clear that rats in the treatment group are capable of both recognizing objects and applying the nonmatching rule. A subsequent decline in the treatment group's performance as the retention interval increases would then likely reflect the increase in memory load demands rather than an inability to remember the nonmatching rule. In sum, DNMS deficits may not necessarily reflect object-recognition impairments, however, there are certain observations that can be made and additional measures that can be included in order to elucidate the cause for the deficit. Nevertheless, maintaining that the one-trial NOP test yields a more straightforward interpretation of treatment effects on

performance because it removes the interpretational problems associated with learning a rule or motivational factors fails to acknowledge that a treatment can also affect a rat's natural exploratory response towards familiar and unfamiliar objects.

Collectively, these findings reveal that interpreting scores in relation to the status of object-recognition memory is more straightforward on the mDNMS task and Go/No-go DNMS task compared to the NOP test. Accordingly, we conclude that these two paradigms provide a useful tool to measure object-recognition memory in rats that avoids the interpretational problems associated with the NOP test.

6.2. Refinements made to the mDNMS task

While testing rats on the mDNMS task, we noticed that there were certain aspects of the task that could be improved on in order to facilitate the interpretation of behaviour as it relates to object-recognition memory (Objective #4). The following section outlines refinements made to the task.

6.2.1. Reducing the potential for positional response biases

The positional biases interfered with estimating rats' object-recognition abilities. It is not uncommon for animals to display positional biases on tests of memory, both at the beginning of training and following training using longer retention intervals (Andrade, Alwarshetty, Sudha, & Chandra, 2001; Cumming & Berryman, 1961; Kangas, Berry, & Branch, 2011; Kangas & Branch, 2008; Mumby et al., 1990; Rothblat & Hayes, 1987). When these positional biases form early on, they are hypothesized to reflect systematic attempts to solve the demands of the task (Krechevsky, 1932). Indeed, prior to learning the reward contingency, a rat may try different strategies to successfully acquire the food reward, and one such strategy may involve always selecting the object on the right. The positional biases disappear as the rat learns a new strategy to successfully retrieve the food reward (i.e., selecting the novel object). In the present thesis these positional biases were also observed during training using longer retention intervals (Chapter 2). When this positional bias appears on trials using longer retention intervals, the rat may be employing this strategy to minimize the demands on memory and decrease the need to store trial specific information over longer retention interval periods. Moreover, the positional bias was transiently displayed on shorter retention intervals that followed sessions using a longer retention interval. This suggests that only after an initial experience with a long delay, this strategy develops (as the rat would not have a priori information on the length of the upcoming

delay). This would mean rats do well on the first trial with a longer delay compared to subsequent trials. Conversely, if it reflects decay followed by the adoption of this strategy, then performance should be worse on the first trial and gradually improve. We observed that the tendency to employ positional biases continued into the sessions that immediately followed ones with longer delays, suggesting that the change to a new delay took time for the rats to adjust to. Removing this habitual behaviour can be difficult, and as evidenced by the rats forming positional biases in the experiments described in Chapter 2 and 3, it can be disruptive to performance. Refining the mDNMS task such that the sample and novel object were no longer positioned next to each other consequently removed the potential for these biases to be formed in the first place (Chapter 5).

6.2.2. Using multiple behaviours to gauge memory

On the mDNMS task performance declined between the criterion sessions during training and subsequent testing on the shortest delay (Chapter 2 and 3). We postulated that the decline in performance might reflect the low cost of making an error (i.e., displacing a sample object) on the test. Indeed, a rat could displace the sample object and then quickly displace the novel object. By placing the sample and novel objects on separate levels in the apparatus, it not only reduced the likelihood that positional biases would form, it also increased the cost of making an error on the test (Chapter 5). Consequently, this modification allowed for the introduction of a new dependent variable to gauge object-recognition: *latency to displace objects* on the test. The findings from the Go/No-go DNMS task revealed that rats had significantly longer latencies to displace sample objects compared to novel objects. Moreover, the rats' latency to displace objects remained consistent between the final criterion sessions during training stage 1 (Figure 5.2c) and subsequent testing (Figure 5.3). Conversely, measuring choice accuracy (displacing only the novel object) revealed that performance declined between training and testing, as rats' tendency to displace sample objects increased during the final stage of testing. Indeed, only three of the nine rats reached the performance criterion within 30 sessions. What we can confirm from these results is that a decline in choice accuracy over time appeared to reflect a change in the rats' *performance*, but not their ability to *recognize* objects, as they continued to show longer latencies to displace sample objects. These findings reveal that when using the Go/No-go paradigm, measuring latency to displace an object provides a more sensitive measure of object-recognition memory than choice accuracy. Moreover, they highlight the advantage of including

an additional behavioural measure to assess object-recognition memory, as relying on only one behavioural measure can mask object-recognition abilities. Lastly, the findings raise an important issue, namely that "errors" on the test may not necessarily reflect poor memory for the sample object—an issue that will be further addressed below in Section 6.5.2.

6.2.3. Using large object sets

On the mDNMS task performance declined between the criterion sessions during training and subsequent testing on the shortest delay (Chapter 2 and 3). We posited that the decline in performance might reflect the use of recurring objects on the task (Appendix B). When new objects were introduced on the test for a group of rats that had received extended testing using the standard object set, rats' accuracy scores improved on average by 15%. This finding revealed that the observed decline in performance in Chapter 2 and 3 may have reflected the use of recurring objects on the task, suggesting that rats had difficulty discriminating between the sample object on the current trial and one encounter several sessions earlier. Based on the findings from the experiment presented in Appendix B, it is unclear why using recurring objects disrupted performance. Using recurring objects may have produced proactive interference on the test or perhaps it caused the objects to become increasingly familiar over time, thus making it difficult for rats to discriminate between two equally familiar objects on the test. In any case, when administering many trials on the mDNMS task it is important to use a large object sets as rats' memory for objects after brief encounters over widely distributed points in time persists for long periods.

Overall, the findings from the experiments described in this thesis reveal that the following features make the mDNMS task a more precise measure of object-recognition memory compared to the existing tasks: 1) using an instrumental response with which the rat makes an explicit choice between familiar and unfamiliar objects, 2) a reward for accurate choices, 3) the possibility of testing individual rats on several trials per session, 4) removing the experimenter from administering individual trials, 5) reducing the potential for positional response biases, 6) using multiple behaviours to gauge memory, and 7) using a large object set.

6.3 Validating the NOP test as a measure of object-recognition memory

An assumption on the NOP test is that the magnitude of the novel-object preference is directly proportional to the strength of the memory for the sample object. Accordingly, an additional goal of this thesis was to compare accuracy scores on the mDNMS task and Go/No-go

DNMS task to preference scores obtained on the NOP test in an attempt to determine whether preference scores on the NOP test accurately reflect object-recognition abilities. The following section describes the results from this thesis in relation to this objective.

6.3.1. Do NOP scores accurately reflect the status of object-recognition memory?

Rats' score on the mDNMS task following the 220-s delay were reliable, indicating that their ability to discriminate which one of two objects had been previously encountered on that trial remained consistent over time (Chapter 2). Conversely, rats' scores on the NOP test using the same delay were not reliable, indicating that their novel-object preference was not consistent across trials. The subsequent correlational analysis performed on the scores from both tasks revealed no significant positive linear correlation. If we accept that the scores on the mDNMS task are an accurate estimation of a rat's object-recognition abilities, and this is what reflects good performance on the task, then the lack of correlation between performances on the two tasks suggests that the magnitude of a rat's novelty preference is not an accurate estimation of its object-recognition abilities. Accordingly, this suggests that both tasks may not tax the same underlying memory processes. It is important to note, however, that correlation estimates more closely approximate the population parameter as sample size increases. Thus, when the sample size is low, as was the case in the experiments, correlations can deviate greatly from the population parameter. Thus, the reported correlations should be interpreted with caution.

After training rats to associate a food reward with a novel object and no food reward with a familiar object, rats exhibited significantly shorter latencies to displace novel objects compared to familiar objects on the test (Chapter 5). This indicates that the rats learned the reward contingency and applied it on the test, revealing that they successfully discriminated between objects on the test, clearly indicating that they recognized the sample objects. The discrimination ratios that were calculated based on the latencies to displace objects on the test did not correlate with rats' investigation ratio scores on the NOP test. Accordingly, these findings reveal that the magnitude of a rat's novelty preference does not correlate with its ability to discriminate between familiar and unfamiliar objects on the Go/No-go DNMS task.

6.3.2. Does a failure to exhibit a novel-object preference reflect an object-recognition impairment?

Similarly, a failure to exhibit a novelty preference did not appear to reflect the status of object-recognition memory. The divergent results on the mDNMS task and NOP test in the

experiments presented in Chapter 3 and 4 further add doubt that the NOP test accurately measures object-recognition memory. Rats successfully discriminated between objects on the mDNMS task following the 72-hr, 3- and ~45-week delay, yet the same rats failed to exhibit a significant novel-object preference following the 72-hr and 3-week delay on the NOP test (Chapter 3). The lack of a novelty-preference following the 72-hr and 3-week delay does not appear to be due to an inability to recognize objects, because the same rats successfully discriminated between objects on the mDNMS task, which was conducted contemporaneously. Thus, we posit that recognition abilities were intact and that the rats' failure to show a novelty preference on the test was due to some alternative explanation. Determining whether the rats failed to exhibit significant novelty preferences on the test following the 72-hr and 3-week delay reflects an inability to recognize the sample objects or some other factor is unknown based on the available data. But, what can be determined from the available data is that on the mDNMS task, the results confirm that the rats did recognize the objects encountered either 72-hr or 3-weeks earlier.

In the experiment presented in Chapter 4, rats in the HPC, PRh, and SHAM group acquired the mDNMS task at the same rate and obtained similar accuracy scores on the test. On the NOP test, however, only rats with HPC lesions exhibited a significant novel-object preference. The fact that PRh and SHAM rats successfully discriminated between sample and novel objects on the mDNMS task suggests that the lack of novel-object preference on the NOP test does not reflect an object-recognition memory impairment. Instead the treatment may have affected some process related to the behavioural expression of this novelty preference, and not necessarily object-recognition memory per se. Taken together these findings reveal that a failure to discriminate between objects on the NOP test should not be uncritically taken as evidence of a failure to recognize objects. Researchers need to be mindful of the limitations of the paradigm, and when reporting results, explicitly acknowledge the non-interpretability of null results.

Collectively, the data from this thesis suggest that 1) the magnitude of the novelty preference is not related to the accuracy of the memory for the sample object, and 2) a lack of novelty preference on the test is not evidence of a failure to recognize objects. Accordingly, we posit that the inconsistencies in the findings on the mDNMS task and NOP test are a reflection of the internal validity problems with the NOP test, and that relying on novel-object preference, as a gauge of object-recognition abilities, will not provide an accurate estimation of the status of

object-recognition memory in rats. Accordingly, the findings reveal that the two tasks may not tax the same underlying construct, viz., object-recognition memory.

6.4. Explaining the divergent results on the mDNMS task and NOP test

The divergent results between rats' performance on the NOP test and the mDNMS task and Go/No-go DNMS task may reflect a difference in the extent to which the tasks tax the same underlying memory process. The following section describes potential alternative mechanisms that may underlie behaviour on the NOP test. In addition, it describes research findings from studies that have used human participants and have raised similar questions pertaining to the validity of novelty preference paradigms as measures of visual recognition memory.

6.4.1. Performance on the NOP test may reflect implicit memory processes

On the NOP test, an increase in novel object investigation may reflect incidental, implicit memory to a greater degree than explicit memory processes. Implicit or nondeclarative memory is characterized as retention for habits, learned motor skills (procedural memory), and priming, whereas, explicit or declarative memory is the retention of information pertaining to facts/general knowledge (referred to as *semantic* memory) and autobiographical events (referred to as *episodic* memory) (Graf & Schacter, 1985; Squire, 1992). Indeed, some researchers posit that novelty preferences reflect implicit memory (Besheer & Bevins, 2000; Desimone & Duncan, 1995; Snyder, Blank, & Marsolek, 2008). Besheer and Bevins (2000) proposed that the NOP test potentially measures response habituation. Habituation is the decrease in responding that occurs as a result of repeated stimulation (Thompson & Spencer, 1966). On the familiarization phase the rat encounters the sample object and investigation towards it decreases as the phase continues. On the test phase, the rat re-encounters the sample object and there is a reduction in the tendency to investigate it as reflected by a preference to investigate the novel object (i.e., response habituation). Conversely, when a rat fails to exhibit a novel-object preference on the test as a result of an increase in sample object investigation, it can reflect a recovery of the habituated response following a period of absence from the sample object which occurs during the delay phase (i.e., response dishabituation). Accordingly, a lack of novelty preference may reflect response dishabituation, despite intact recognition. Because the behavioural measure used on the NOP test can reflect either recognition or habituation processes, when there is a lack of novelty preference after some form of treatment, it makes it difficult to interpret which of the two underlying processes a treatment may have exerted its effect on. Alternatively, on DNMS tasks,

the behavioral measure consists of a rat making an explicit choice response based on recognition of the sample object, and thus, interpreting performance is not confounded by the potential for response habituation.

When using varying delay intervals on the NOP test (e.g., lasting minutes to hours), and when a rat then exhibits a novelty preference following the shortest delay but not following longer delays, it is presumed this failure to preferentially investigate the novel reflects the forgetting of the sample object (Ennaceur, Cavoy, Costa, & Delacour, 1989; Puma & Bizot, 1998; Puma, Deschaux, Molimard, & Bizot, 1999). There could, however, be alternative explanations for these findings, such as the rat simply regains interest in the sample object after not encountering it for long periods of time and has not necessarily forgotten it. This increase in sample object investigation on the test may reflect *stimulus satiation* (Glanzer, 1953, 1958). When an animal encounters a stimulus, a quantity of satiation for that stimulus develops, resulting in a reduction in the animal's tendency to respond to that stimulus when it re-encounters it. When the stimulus is no longer present, the quantity of satiation dissipates. This concept can explain recovery in interest in the stimulus following the absence of it. On the basis of this, novelty preference can be high following a very brief delay between the sample and test phase, whereas after a longer delay, the reintroduction of the stimulus (sample object) produces an increase in responding to it. Accordingly, this would decrease investigation towards the novel stimulus, consequently resulting in a null preference.

Evidence from experimental approaches other than those relying on behavioural measures also lend support to the idea that performance on the NOP test may reflect implicit memory processes. Experiments assessing the role of the perirhinal cortex (PRh) in object-recognition memory using immunohistochemical methods that analyze the activation of immediate early genes (IEGs), such as c-*fos,* have been used as indirect measures of neuronal activity. Indeed, measuring the levels of Fos, the protein product of c-*fos*, has revealed that there is increased Fos-immunoreactivity (IR) in the PRh following visual presentations of novel stimuli, and a decrease in Fos-IR following presentations of familiar stimuli (Albasser, Poirier, & Aggleton, 2010; Zhu, Brown, McCabe, & Aggleton, 1995; Zhu, Brown, & Aggleton, 1995; Zhu, McCabe, Aggleton, & Brown, 1996, 1997). These findings are consistent with electrophysiological data in nonhuman primates (Fahy, Riches, & Brown, 1993; Miller, Gochin, & Gross, 1991) and rats (Zhu, Brown, & Aggleton, 1995) revealing a decreased response in

neurons in the PRh and area TE following repeated presentations of the same visual stimulus. These findings suggest that neurons in the PRh respond less to visual stimuli that were previously encountered. This phenomenon, referred to as *repetition suppression*, has been hypothesized to underlie novelty preferences in visual selective attention (Desimone, 1996). Accordingly, some researchers have argued that increased looking times towards a novel stimulus may not necessarily require explicit memory processes whereby the presentation of a stimulus is matched to an internal representation or memory trace of the previously encountered stimulus (Snyder et al., 2008). These studies reveal that when subjects passively view either novel or familiar stimuli, c-*fos* expression in the PRh is influenced by exposure to novel stimuli. While these results hint that the PRh is involved in learning-related neuronal plasticity, it is not necessarily the case that the PRh is *critical* for object-recognition memory.

6.4.2. We are not alone: The same concerns have been raised regarding the internal validity of the human visual-paired comparison task

Human infant and animal research both have similar challenges when it comes to designing an experiment to assess memory, in that the experimenter cannot depend on the subject's ability to comprehend and use language. The *Visual Paired-Comparison* (VPC) paradigm (the human and nonhuman primate analogue of the NOP test) suffers from the same inherent limitation when interpreting the significance of a null preference on the test. Briefly, there are two phases on the VPC task: a familiarization and test phase. On the familiarization phase, the participant is provided a designated amount of time to look at a stimulus object (typically on a computer screen). Following a retention interval it receives a test, whereby the familiar stimulus is now presented next to a novel one for a period of time. The dependent measure is the amount of time spent looking at each stimulus object on the test. When a participant exhibits a significant bias to look at the novel stimulus, it is inferred that the participant recognizes the sample stimulus. It is not uncommon for researchers to posit that a null preference reflects forgetting of the sample stimulus (Sophian, 1980). This is especially true when working with human infants or nonhuman primates that do not possess the language skills to communicate, so researchers must solely rely on the amount of time the subject spent looking at a stimulus to determine if the participant recognizes the sample stimulus (unlike human adults who can verbalize whether or not they recognize the stimulus). In the past few decades, researchers studying cognitive development in human infants have raised concerns regarding the

typical interpretation of null results on novelty preference tasks (Bahrick & Pickens, 1995; Richmond, Colombo, & Hayne, 2007; Sophian, 1980).

Preference to attend to novel stimuli can change as a function of the delay between the familiarization and test phase for reasons that are not directly related to the strength in memory for the original stimulus (Bahrick, Gogate, & Ruiz, 2002; Bahrick, Hernandez-Reif, & Pickens, 1997; Bahrick & Pickens, 1995; Berlyne, 1957; Courage & Howe, 1998; Richmond, Colombo, & Hayne, 2007b; Spence, 1996). Using the VPC task, researchers found that 3-month old infants showed a novelty preference when the delay between the familiarization phase and test phase was short (1 min), but failed to exhibit a significant preference following both a 24-hr and 2-week delay (Bahrick & Pickens, 1995). The researchers included a fourth, 1-month long delay, and intriguingly found that the infants showed a significant preference to look at the *familiar* stimuli. The findings on both the shortest and longest delay clearly indicated that the infants recognized the familiar stimuli because they exhibited a significant bias to attend to one stimulus. Based on the results from the intermediate delays alone, one would be unable to conclude whether or not the infants recognized objects following a 24-hr delay, however, the results from the 1-month delay confirmed that they could. The lack of a preference following both the 24-hr and 2-week delay reveals that the performance on the test may not reflect memory per se, and instead may reflect some other factor (e.g., attentional processes). According to Bahrick and colleagues (1997), the degree of novelty preferences reflects how "accessible" the memory trace is at the time of test (p. 4). Following short delays (e.g., minutes or hours) between the familiarization phase and test, the memory trace is highly accessible, revealing no need to attend to the familiar stimulus. Conversely, following very long retention intervals (e.g., months) the memory for the sample is less accessible, thus, the individual requires more time to look at the sample stimulus in order to determine if it was previously encountered. Between short and long delay periods there is an intermediate phase whereby the subject attends equally to the novel and familiar stimulus as there is a shift in the accessibility of the memory trace. According to this theory, null preferences do not necessarily reflect forgetting, rather they reflect a competition in attentional processes towards novel and familiar stimuli (Bahrick et al., 1997).

Previous studies have found that both humans (Gross, Hayne, Herbert, & Sowerby, 2002; Pascalis, Hunkin, Holdstock, Isaac, & Mayes, 2004; Richmond et al., 2007; Wilk, Klein, & Rovee-Collier, 2001) and nonhuman primates (Bachevalier, Beauregard, & Alvarado, 1999;

Nemanic et al., 2004; Pascalis & Bachevalier, 1999) fail to exhibit novelty preferences on the VPC task despite exhibiting intact recognition on paradigms that incorporate an instrumental response. Richmond et al. (2007) conducted an experiment whereby different groups of adults were tested using the same stimuli on either the VPC task or a forced-choice recognition task using delays lasting 3 min, 24 hr, 1 week, 2 weeks, 6 months and 12 months. On the VPC task, the groups of participants exhibited significant novelty preferences on delays lasting between 3 min and 2 weeks, a null preference on the 6-month delay, and the group tested using the 12-month delay exhibited a significant sample stimulus preference. On the forced-choice recognition task, on the choice phase when two stimuli were presented (one familiar and one novel), the participants were instructed to press the response button that corresponded to the stimulus on the screen (left or right) that was previously encountered. When testing participants on the forced-choice task, the percent of participants that accurately identified the familiar stimulus was significantly above chance following all delays (scores ranged from 90% on the 3-min delay to 75% on the 12-month delay). Accordingly, on the VPC task, adults failed to exhibit a novelty preference following the 6-month delay, however, on the forced-choice task adults who were tested using the same stimuli exhibited accuracy levels that were significantly above chance (75%), indicating stimulus recognition. The researchers concluded that the participants tested on the VPC task likely recognized the stimuli from 6 months earlier, but that relying on novelty preferences alone does not provide an accurate estimate of recognition abilities. These findings reveal that novelty preference scores do not necessarily relate to the accuracy with which an individual can explicitly state whether or not they recognize a previously encountered stimulus. This suggests that the VPC task does not provide an accurate estimate of object-recognition memory, which raises concerns regarding the internal validity of it as a measure of object-recognition memory.

As a final note, it is important to explain the rationale for drawing a comparison between humans and animals. It is not to argue that there is a specific functional similarity between the decline in novelty preferences over long periods, but rather to highlight the importance of being cautious interpreting a lack of novelty preference as an object-recognition deficit. These findings are especially important in relation to administering some form of treatment and then testing rats on the NOP test using varying delays. When rats' exhibit an intact novelty preference following a short delay, but fail to do so following longer delays, it is taken as evidence of a treatment

effect on memory processes. While these results reveal that the treatment has not abolished a rat's ability to express a novelty preference, it does not confirm with the same level of certainty that the lack of novelty preference on the longer delay reflects an inability to recognize the sample object. In summary, changes in behaviour (i.e., novelty-preferences) as a result of prior exposure may be based on recognition memory, but because recognition memory is not the only process producing the response, it should not be assumed with certainty that the observed behaviour reflects the degree of recognition, especially considering that there are alternative candidate explanations.

6.4.3. A method to clarify the divergent results on the mDNMS task and NOP test

One way we could have indirectly determined whether performance on the DNMS and NOP test reflects two dissociable memory processes would have been to implement a distraction during the delay period on both tasks (e.g., by introducing new objects). In humans, when distractions are introduced during the retention interval between the learning and test phase, such as counting numbers backwards in sets of 6, it disrupts performance on tests designed to assess explicit memory, but not on tests of implicit memory (Graf & Schacter, 1987). The distraction is thought to produce *retroactive interference*—newly acquired information interferes with the ability to recall previously learned information. Previous research using nonhuman primates has shown that implementing distractions during the retention delay, by presenting different objects, disrupts performance on DNMS tasks (Zola-Morgan & Squire, 1985; Zola-Morgan, Squire, & Amaral, 1989a, 1989b). This is thought to occur because the objects encountered during the delay interfere with processing sample-object information. Previous experiments in our lab have shown that DNMS performance is disrupted in rats when random objects are presented during the delay period (D. Mumby, personal communication, 2012). This suggests that the underlying memory processes that rats use when performing the DNMS task resemble those that humans use when they perform explicit-memory tasks (i.e., retaining information for the sample object over the delay). This is important because it suggests the DNMS task is not a measure of implicit learning, a form of memory that is not impaired in patients who have sustained damage to the medial temporal lobes (Cohen & Squire, 1980; Milner, Corkin, & Teuber, 1968; Warrington & Weiskrantz, 1968, 1974). It suggests that successful performance on DNMS tasks likely involves neural substrates similar to those damaged in medial temporal lobe amnesia. Accordingly, to elucidate whether both the mDNMS task and NOP test tax similar memory processes (i.e.,

explicit or implicit), it would be important to conduct an experiment whereby rats encounter random objects during the delay period on both the mDNMS task and NOP test to determine whether this distraction disrupts subsequent performance on the test. If novelty preference scores do not decline on trials with distractor objects relative to trials without distractor objects, but they do on the mDNMS task, then it could provide indirect evidence that the two tasks do not measure similar memory processes.

6.5. Interesting observations on the mDNMS task that deserve further attention

This section briefly describes some interesting observations that were made using the mDNMS task, and their theoretical implications.

6.5.1. The lack of a delay-dependent decline on the mDNMS task

An observed phenomenon on working-memory tests is that as the retention interval increases, accuracy on the test declines. This is hypothesized to reflect a decay in the memory trace that occurs over time between the encoding of the sample object during learning and the test (Brown, 1958; Tulving, 1972). Theoretically, the scores on the mDNMS mixed-delay testing (Chapter 2) should have declined as a function of the delay length. However, there was no significant difference in scores between the 100 and 630-s delay. The results from Chapter 2 lend support to a behavioural perspective theory of memory proposed by White (2002). In a clever experiment, Sargisson and White (2001) varied the retention interval used to train pigeons on a delayed matching-to-sample task (on this variant, selection of the *sample* stimulus is rewarded). Pigeons received extensive training on the task using one of several different delays (e.g., 0, 2, 4, or 6 s), and then were tested using delays ranging from 0 to 10 s. The researchers found that accuracy at each delay varied as a function of the delay-length used during training. Pigeons trained using a 0-s delay displayed the typical delay-dependent decline in accuracy with increasing delays, with the highest scores on the 0-s delay. Conversely, pigeons trained using a 4-s delay exhibited a more flattened slope in accuracy scores, with the highest levels of accuracy displayed on the 4-s delay. Moreover, their accuracy scores on the 0-s delay were slightly lower compared to the pigeons trained using a 0-s delay. Thus, accuracy in discriminating between two stimuli was specific to the length of the delay used during training. These results are contrary to what the decay theory would propose in terms of memory processes—that the representation (memory trace) of the stimulus that was encoded during learning gradually fades as time passes on. According to White (2002), “objects or events can be discriminated at a temporal

distance…[and]…information used for the discrimination is specific to the time of remembering" (p. 141-142). The information that is used to make a choice between stimuli is based on "the value of the stimulus effect…[and the animal's]…history of learning about rewards" (p. 143). This behaviourist model proposes that memory is not based on the formation of a memory trace that is retrieved at the time of testing, but rather it is based on the reward contingency and the reinforcement history for similar choices in the past. In the experiment presented in Chapter 2, rats were trained using a 30-s delay, thus, it may explain the flattened gradient observed between the delays from 100 s to 630 s. Perhaps, if rats were trained using a shorter retention interval (e.g., 5 s) we would have observed the typical delay-dependent decline. In any event, the findings provoke similar questions about measuring working-memory in nonhuman animals; namely, whether performance on the task reflects certain theoretical processes such as decay theory.

6.5.2. Distinguishing between learning/memory and performance: Errors do not necessarily reflect impaired object-recognition

We observed a decline in performance after introducing a new delay during mixed-delay testing (Chapter 2) and after administering probe tests (Chapter 2 and 3). These findings reveal that a decline in accuracy does not necessarily reflect the rat's memory abilities but may instead reflect a reaction to a change in procedure that the rat had become accustomed to (Honig & Wasserman, 1981), or its incentive to respond accurately after receiving probe tests that alter the reward contingency (Honig & Dodd, 1986). Additionally, when we measured latency to displace an object on the Go/No-go DNMS task (Chapter 5), it was clear that all of the rats recognized the sample object, however, measuring 'choice accuracy' presented a different picture. In this case, it appeared as though the majority of the rats failed to discriminate between the novel and sample objects on the test. These findings reveal the importance of recognizing that errors on a test do not necessarily reflect impaired recognition and they highlight the importance of designing a task that limit these interpretational problems from occurring.

6.6. Addressing the shortcomings of the mDNMS task and Go/No-go DNMS

The primary goal of this thesis was to develop a new object-recognition task that addressed the known limitations of the existing tasks. Inherent to any behavioural task, the mDNMS task also had drawbacks. Some limitations associated with using the mDNMS task and the refined Go/No-go DNMS task include: 1) issues controlling the delay length 2) the method

used to administer probe tests, 3) the pre-training procedure, and 3) the time requirements to collect data. These drawbacks are discussed below.

6.6.1. Controlling the delay length

Using a large open space for the delay area whereby the rat could walk around freely was an important feature of the mDNMS task when implementing retention intervals that lasted longer than a minute. On the conventional DNMS task apparatus, when rats are confined to a small area during the delay they can become agitated and make hasty responses at the end of the delay. Thus, the goal on the mDNMS task was to increase the area in which rats could spend time moving around, and feel less constrained. However, this presented a problem when trying to assess memory for objects following a very short retention interval. In the experiment described in Chapter 2, the minimum average delay when training rats on the mDNMS task was ~30 s, and was similar in the other experiments. Then, after rats received training using gradually longer delays, the average length of the shortest delay increased to ~100 seconds. Consequently, when scores declined during mixed delay testing compared to the final criterion sessions, it was unclear whether this decline reflected an increase in the demands on memory for the sample objects or some other factor, such as proactive interference due to an increase in the number of times objects were encountered (or a combination of factors). Ideally, any task designed to test object-recognition memory includes the option to measure memory for an object following a very brief retention interval. This is because it provides an index of memory when the lowest demands are placed on it. This is an important feature because performance on DNMS tasks following some experimental manipulation can produce impairments following delays lasting as short as 15 s, but not on delays lasting 4 s (Mumby & Pinel, 1994; Mumby, Pinel, et al., 1995; Wiig & Bilkey, 1995). Accordingly, not being able to enforce a short delay on the mDNMS task means the experimenter could potentially miss out on observing intact recognition abilities following short delays whereby the treatment may not exert its effects.

Moreover, another potential issue is the variability in delay length across sessions both within and between individual rats when testing using a particular delay. This specifically presents problems when trying to administer sessions with no set delay (e.g., the minimum delay). For example, the rat could end the sample phase and quickly run to the choice phase (e.g., 10 s), but on other sessions the rat may take its time, resulting in longer retention intervals (e.g., 100 s). The variability observed across sessions for individual rats can be a potential problem in

terms of a source of random error when trying to measure test re-test reliability. This could present a problem when trying to determine whether rats' scores on the test are reliable. In terms of between-subjects variability this lack of control over delay length only presents as a problem if the goal is to determine object-recognition abilities following a set delay (e.g., 30 s). If the goal is simply to determine accuracy scores following some undesignated short or long retention interval, then it presents less of a problem. In any case, not being able to control the delay can present a problem when trying to minimize the variability between rats (and within sessions for a particular rat). One way we could modify this in future experiments would be to train rats to associate a cue (e.g., noise) that would signal the end of the dely.

6.6.2. The method used to administer probe tests

The overall findings from the probe tests revealed that rats were not relying on olfactory cues to correctly locate the food reward. Interpreting the probe test results was difficult, however, for the experiments described in Chapter 2 and those of the PRh rats in Chapter 4. Scores on the probe tests and normal tests were not significantly different from one another, however scores on both the probe tests and normal tests were not significantly different from chance. Administering probe tests modifies the characteristics on the task, which consequently, can disrupt performance on the test despite intact recognition abilities (Honig & Dodd, 1986). We suspect that introducing these changes to the reward contingency during probe testing was disruptive to rats' performance on the probe tests and the normal tests that were administered contemporaneously. Accordingly, the decline in task accuracy during probe testing may have reflected the rat's incentive to respond accurately, and not necessarily its memory abilities or the ability to detect the odor of the reward. In the future, a better design would involve baiting both the sample and novel objects with a food reward on the test, but only having the reward underneath the novel object accessible, thus eliminating the need to conduct probe tests.

6.6.3. The pre-training procedure

On the Go/No-go paradigm it was found that latency to make a choice on the test was a more sensitive measure of a rat's memory for the sample object compared to choice accuracy (Chapter 5). One reason why rats may have continued to displace sample objects on the test, despite exhibiting longer latencies to displace them, may be a reflection of the training method that was used. During training the rats were reinforced for displacing the same sample object over multiple trials on the sample phase, thus it makes sense that they would predict to find a

reward underneath the sample object on the test phase. Accordingly, perhaps providing multiple opportunities to collect a reward after displacing a particular sample object *impeded* rats' ability to acquire the reward contingency (i.e., nonmatch to the sample on the test). Indeed, this is reflected in latency scores on the first five sessions during training stage 1 whereby rats exhibited a shorter mean latency to displace objects on the No-go trials compared to Go trials (Figure 5.2c). The training procedure was adapted from the one used on the mDNMS task. Thus, it raises the question as to whether this training method interfered with performance on the test, rather than facilitated performance. An important future experiment would include modifying the training method to include only one opportunity to displace a sample object before encountering it on the test. This would help determine the ideal training method that would lead to faster task acquisition and/or a reduced tendency to displace sample objects on the Go/no-go DNMS task.

6.6.4. Time requirements to test rats

Rats acquired the mDNMS task in significantly fewer trials compared to conventional DNMS tasks. However, the mDNMS task still requires a considerable number of weeks to conduct an experiment, as does the Go/No-go DNMS task. Administering more than one session per day and testing rats daily, however, would reduce the length in time required to collect data. Regardless, compared to using the NOP test, the mDNMS task is less practical in that it will require more time and effort to conduct an experiment. However, given the concerns about internal validity when the NOP test is used to make inferences about object-recognition abilities, the choice of which task to use in a memory experiment would appear to be a choice between getting dubious data quickly versus taking a bit more time in order to get high-quality data.

6.7. Future Directions

The almost exclusive reliance on novelty-preference paradigms to study object-recognition in rats needs to be replaced (or at least supplemented) with procedures that yield data that are easier to interpret. Relying on the preference to investigate an object provides limited information on the status of object-recognition abilities, and worse, can potentially lead to misinterpretations of the status of object-recognition memory. This is especially the case when null preferences are taken to reflect an object-recognition impairment. It is unlikely that researchers will abandon using novelty-preference paradigms considering the ease and speed that data collection can be done. Indeed, the field of behavioural neuroscience relies too much on using quick and easy behavioural tests to measure complex cognitive processes. Extremely

sophisticated techniques have been developed to examine the biology of the nervous system, yet there is an over-reliance on using simple behavioural tasks to answer research questions (Cahill, James, & Weinberger, 2001; Krakauer, Ghazanfar, Gomez-marin, Maciver, & Poeppel, 2017; Peters, Pothuizen, & Spruijt, 2015; Sarter, 2004; Spruijt, Peters, Heer, Pothuizen, & Harst, 2014).

Some researchers have acknowledged the limitations of the NOP test and have developed clever new object-recognition tests in response to these limitations. These tasks were designed to address the limitations related to the low number of trials that can be administered when using standard open field arenas and the stress that can be caused to the rats by handling them between phases (Albasser, Chapman, et al., 2010; Ameen-Ali, Eacott, & Easton, 2012). On both tasks, the rat is rewarded with a food pellet that is placed under each object when it investigates them during each phase. The food is used to encourage rats to investigate the objects and to shuffle back and forth between trials in the apparatus. The advantage of these paradigms is that they allow for the collection of many trials within a session and the rat is not handled. However, unlike the DNMS task, the food reward is not contingent on the selection of the novel object first, as there is a food reward placed under the sample too. Thus, this paradigm still relies on measuring the amount of time the rat spends exploring familiar and unfamiliar objects. Consequently, this paradigm still suffers from the same inherent limitations of the NOP test, despite adjusting for some of the shortcomings.

The findings of the present thesis raise important implications in relation to the low reproducibility of behavioural neuroscience results (Bailoo, Reichlin, & Würbel, 2014; Bespalov & Steckler, 2018; Spruijt et al., 2014; Voelkl & Würbel, 2016) and the low predictive validity of experiments focused on developing pharmacological treatments for neurological and psychological disorders (Cummings, Morstorf, & Zhong, 2014; Kola & Landis, 2004; Mcgonigle & Ruggeri, 2014). Reproducibility depends on experimental designs that have both high internal and external validity. Factors pertaining specifically to experimental design and analysis[12] that have been described as contributing to low reproducibility of research findings are: low statistical power, lack of randomization, experimenters not blind to group allocation, and lack of

[12] Indeed, other recognized contributing factors unrelated to experimental design include publication bias (MacLeod, 2011; Scargle, 2000), poor description of experimental procedures (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010), and standardization of animal housing environments across laboratories (Voelkl & Würbel, 2016; Würbel, 2001), to name a few.

standardization of experimental methods across laboratories (Button et al., 2013; Gulinello et al., 2018; Ioannidis, 2005). Surprisingly, a crucial factor pertaining to experimental design that has not been discussed in detail is whether or not the behavioural task being used in the experiment is, in fact, a valid measure of the internal construct under investigation (Sarter, 2004). Ultimately, low reproducibility and poor predictive validity likely reflect a combination of known and unknown factors, but one factor that is missing extensive research on, is the validation of behavioural paradigms used in the approach to study underlying neural mechanisms in animals.

The internal validity problems associated with the NOP test as a gauge of object-recognition memory are undeniable, along with the lack of research that has focused on assessing its internal validity. Perhaps this reflects a form of *pluralistic ignorance*, whereby the majority of researchers genuinely believe the NOP test has internal validity problems yet assume, incorrectly, that others are not thinking the same thing. This may be a result of a lack of conversation on this topic. In this case, more discussions are needed on validating novelty preference paradigms and using suitable behavioural paradigms to measure internal constructs using animal models. Reducing the disciplinary disconnect between the field of neuroscience and ethology would be beneficial, as would familiarizing oneself with articles published in journals that have a focus on animal behaviour (Olsson, Nevison, Patterson-kane, & Sherwin, 2003; Peters et al., 2015). Ultimately, designing robust behavioural tasks that allow for the collection of meaningful data requires an understanding of animal behaviour, an awareness of potential confounding variables, and patience.

6.8. Conclusions

The findings from this thesis reveal the utility of the mDNMS task and the internal validity problems associated with using the NOP test as a means to measure object-recognition memory in rats. Moreover, the refinements made to the mDNMS task yielded more sensitive measures of object-recognition memory while eliminating drawbacks in the initial design. This thesis established that a lack of novelty preference on the NOP test does not reflect an inability to discriminate the familiarity of a previously encountered object. Moreover, novelty-preference scores do not appear to relate to the strength in memory for a sample object as evidenced from the correlational findings using both the mDNMS and Go/No-go DNMS task. These results raise concerns about the way NOP data are typically interpreted.

Collectively, the findings from this thesis reveal important factors that should be included when designing behavioural tasks used to infer memory processes in rats: 1) an instrumental response with which the rat must make an explicit choice between familiar and unfamiliar stimuli, and a reward for accurate choices, 2) the option to measure multiple behaviours in order to increase the sensitivity of the task, and 3) the minimization of the number of alternative explanations for observed behaviour of the task. Ultimately, it allows for a clearer interpretation of behaviour as it relates to the particular defined construct under investigation. In summary, the findings from this thesis reveal that there are clear pitfalls when it comes to assessing object-recognition memory in rats. However, by being mindful of potential shortcomings when designing a task and being vigilant when interpreting behaviour on the task, it can reduce interpretational problems, ultimately providing a more sensitive estimate of the construct under investigation.

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Appendix A

Table 1.

Results from studies using DNMS to assess object recognition in rats

Study	No. of pre-training trials	DNMS acquisition				Testing at longer delays	
		Performance criterion	Delay (s)	Mean score on criterion trials	Mean no. of trials to reach criterion	Delay & approx. mean score on test	Mean no. of training trials
Thesis Experiment 1 (Chapter 2)	**74.68**	**16/20**	**30***	**84%**	**24.44**	**100 s: 67% 220 s: 59% 330 s: 66% 630 s: 63%**	**None 32 64 52**
Thesis Experiment 2 (Chapter 3)	**156.56**	**16/20**	**69***	**81%**	**79.44**	**72 hr: 67% 3 wk: 70% 45 wk: 64%**	**None**
Thesis Experiment 3 (Chapter 4)	**120**	**16/20**	**34***	**81%**	**79**	**n/a**	**n/a**
Aggleton, 1985 (Experiment 1)	None	40/50	0	81%	130	20 s: 84% 60 s: 74% 120 s: 80%[a]	None
Aggleton et al., 1986	None	40/50	0	>80%	190	20 s: 82% 60 s: 72%	None
Rothblat & Hayes, 1987	240-480	27/36	10	77%	177.6	30 s: 70% 120 s: 63%	None
Mumby et al., 1990	100	21/25[b]	4	90%	235	15 s: 91% 60 s: 81% 120 s: 77% 600 s: 57%	Max. 200 ” ” 100
Mumby et al., 1992	100-125	17/20[b] or 21/25[b]	4	>85%	280	15 s: 89% 60 s: 79% 120 s: 76% 600 s: 63%	125 -250 ” ” ”
Kesner et al., 1993	8	45/60	1-4	83%	76	10 s: 78% 20 s: 73%	Max. 60 Max. 260

Mumby et al., 1993 (Experiment 1)	41.7	17/20[b]	4	>85%	340	15 s: 75% 30 s: 79% 60 s: 72% 120 s: 71% 300 s: 68%	160 ” ” ” ”
Mumby et al., 1993 (Experiment 2 pre-surgery data)	55	17/20[b]	4	>85%	336	15 s: 88% 60 s: 82% 120 s: 73% 300 s: 67%	Max. 160 ” ” 80
Wood et al., 1993 (Experiment 1)	Max. 125	21/25[b]	4	>85%	350	15 s: 80% 30 s: 81% 60 s: 78% 120 s: 74% 300 s: 68%	200 ” ” ” ”
Wood et al., 1993 (Experiment 2 pre-surgery data)	Max. 125	17/20 [b]	4	>85%	347	15 s: 88% 30 s: 78% 60 s: 80% 120 s: 70% 300 s: 66%	Max. 160
Mumby & Pinel, 1994 (Pre-surgery data)	125	17/20[b]	4	>85%	420	15 s: 87% 60 s: 76% 120 s: 72% 600 s: 58%	Max. 160 ” ” Max. 80
Mumby, Mana, Pinel, David, & Banks, 1995	100	17/20[b]	4	>85%	280	15 s: 82% 30 s: 82% 60 s: 83% 120 s: 82%	160 ” ” ”
Mumby, Pinel, Kornecook, Shen, & Redila, 1995	88	17/20[b]	4	>85%	360	15 s: 82% 30 s: 77% 60 s: 77% 120 s: 76%	120 ” ” ”
Wiig & Bilkey 1995	75	26/30	4	>86%	173.6	15 s: 79% 30 s: 79% 60 s: 72% 120 s: 66%	50 ” ” ”
Mumby et al., 1996 (Study 1 pre-surgery data)	100	17/20[b]	4	>85%	316	15 s: 88% 60 s: 81% 120 s: 76% 300 s: 75%	120 ” ” ”

Duva et al., 1997 (Experiment 1 pre-surgery data)	60	17/20[b]	4	>85%	264	60 s: 74% 120 s: 67% 300 s: 69%	Max. 100 ” ”
Duva et al., 1997 (Experiment 2)	75	17/20[b]	4	>85%	334	60 s: 77% 120 s: 67% 300 s: 68%	Max. 100 ” ”
Kornecook et al., 1999 (Pre-surgery)	125	17/20[b]	4	>85%	395	15 s: 91% 30 s: 83% 60 s: 78% 120 s: 74%	Max. 120 ” ” ”
Clark et al., 2001	44	32/40	4	>80%	220	30 s: 75% 60 s: 76% 120 s: 78% 60 s: 82%[c]	None ” ” 387[c]

Note: Mean number of trials to reach criterion exclude the criterion trials. The referenced articles are listed in chronological order. For studies that included treatment groups, only the data for the SHAM (control) group are reported.

*Delay during final five criterion sessions

[a]Score is based on 30 trials instead of 50

[b]Rats were required to reach this criterion on two consecutive occasions

[c]Results when rats received extended training with a 60-s delay

Appendix B: Does a decline in mDNMS task performance over time reflect the use of recurring objects?

Introduction

The *delayed nonmatching-to-sample* (DNMS) task is considered to assess *working-memory*—memory for information that is only needed for one trial on a task (Honig, 1985). A key factor that made the nonhuman primate DNMS task successful, compared to the nonspatial delayed-response tasks devised in the 1950s and 1960s, was the use of a large set of stimulus objects (Gaffan, 1974; Mishkin, 1978; Mishkin & Delacour, 1975). Indeed, the nonspatial delayed-response paradigms in the 1950s and 1960s used only a small set of recurring objects across trials, resulting in the stimuli becoming familiar after only a few trials (Correll & Scoville, 1965; Etkin & D'Amato, 1969; Mishkin & Weiskrantz, 1958; Scheckel, 1965; Weinstein, 1941). Consequently, this made the task a measure of *recency memory* (i.e., which of these equally familiar stimuli did you see most recently?), which nonhuman primates have difficulty successfully performing. Accordingly, to ensure the DNMS remains a test of *recognition memory*—the ability to detect the familiarity of a previously encountered item— a large pool of stimulus objects is required to ensure objects do not repeat often, and consequently, do not become overly familiar. Anywhere between 300 and 1300 objects have been used on the nonhuman primate DNMS (cf. Aggleton & Mishkin, 1983; Meunier, Bachevalier, Mishkin, & Murray, 1993; Murray & Mishkin, 1986; Zola-Morgan, Squire, & Amaral, 1989; Zola-Morgan & Squire, 1985) and anywhere between 250 and 400 objects have been used on the rodent DNMS paradigms (cf. Mumby, Pinel, & Wood, 1990; Rothblat & Hayes, 1987).

In the present thesis a large stimulus set was used (e.g., 192 objects during the final stage of mDNMS testing) in an attempt to reduce the number of times a particular object recurred over trials[13]. The objects began to repeat by trial 96 (day 24 of testing, as there were 4 trials per day). Comparatively, on Rothblat's and Hayes's DNMS version, 250 objects were used, which began to repeat by trial 126 (day 10 of testing, as there were 12 trials per day), and on Mumby's version 350 objects were used which began to repeat by trial 176 (day 7 of testing, as there were 25 trials per day). Despite using recurring objects, previous studies reported that rats' accuracy on the final criterion sessions during training and subsequent testing using the same delay remained around the same asymptotic level of performance. When rats were trained and tested using a 10-s delay their scores remained around 76% (Rothblat & Hayes, 1987) and when using a 4-s delay

[13] In the experiment presented in Chapter 3, 42 objects were used as samples on the ~45-week delay, so the total number of objects used for the 80-s delay was 150, not 192. Thus, there were only 75 object pairs.

scores remained around 90% (Mumby et al., 1990). Conversely, on the mDNMS task performance declined between the criterion sessions during training and subsequent testing on the shortest delay. The mean accuracy score on the five criterion sessions in the experiment described in Chapter 2 was 84% and as testing continued, mean scores on the shortest delay dropped to 67%. As described in Chapter 2, we suspected that the discrepancy in scores between training and testing likely reflected an effect of the different testing procedures on *performance* more so than on memory. Rats received several successive sessions with the same delay and then it changed to a new one. Over several successive sessions with the same delay length, certain aspects of performance become habitual, and the change in procedure that occurred when the delay was changed was enough to transiently disrupt performance (as also evidenced during training with longer delays—see Figure 2.2a). It is also possible that the decline in performance reflects the increase in the shortest delay from 30 s during training to 100 s during testing. Thus, the decline in accuracy may reflect an increase in the demands on memory considering the minimum delay length became longer. However, this same decline in performance between the final five criterion sessions and later testing also occurred in the experiment presented in Chapter 3 (81% to 65%), despite no abrupt changes in the delay length between sessions nor a major increase in the minimum delay length between the training and testing stages (69 s to 82 s).

One potential reason for the decline in performance from training to resting may reflect the use of recurring objects on the task. On the mDNMS task, by the end of testing using the shortest delay, rats encountered a particular object on average six (Chapter 2) and five times (Chapter 3). Conversely, in the Rothblat and Hayes experiment rats encountered each object a maximum of two times, as they only administered an average of 238 trials. Thus, on the mDNMS task, rats encountered object more often over time, and this may have led to the objects becoming increasingly familiar. However, Mumby and colleagues provided extensive training using several different delays, resulting in the rats encountering a particular object at least six times by the end of testing (Mumby et al., 1990). Thus, the *number* of encounters with a particular object alone may not explain the decline in performance on the test. However, a factor that was different on the mDNMS task compared to conventional DNMS tasks was the *amount of time* a rat could spend investigating a particular object on each trial. On conventional DNMS tasks the experimenter removes the object from the apparatus immediately after the rat displaces it from over the food well. This means the rat spends ~1-2 s investigating an object per trial.

Interestingly, when rats are given additional time to investigate the sample object, DNMS performance improves (Beck & Kalynchuk, 1992), suggesting that more time spent investigating the object (encoding object features) improves retention of object information. On the mDNMS task there is no restriction in the amount of time that a rat can spend investigating an object on both the sample and test phase. Based on our observations, rats would spend time investigating an object both before and after displacing it from over the food well. On the sample phase rats would spend, on average, ~6 s investigating a sample object. Thus, perhaps the amount of time rats spent investigating objects over repeated sessions caused the objects to become increasingly familiar over time, making it difficult for the rats to discriminate between the sample on the current trial and the "novel" object. This may explain why performance was initially high on the five criterion sessions during training, as a particular object had only been encountered once or twice at this point, and then declined during subsequent testing with repeated exposures to the objects.

The extent to which a rat would have difficulty applying the nonmatching rule on the test for a sample object it encountered minutes earlier and a "novel" object it encountered 64-96 trials (16-24 sessions)[14] earlier is unknown. However, given that on the mDNMS task rats were capable of recognizing objects that they encountered ~45 weeks earlier (Chapter 3), it is not implausible to consider that prior brief exposures to an object can cause it to become so familiar that on the test that a rat has difficulty distinguishing between it and an object encountered minutes earlier on the sample phase. The goal of this experiment was to determine if accuracy scores would improve in a group of rats when using new objects were compared to the standard objects that the rats already had prior encounters with. Once we finished the experiment described in Chapter 3 we gave the rats trials on the mDNMS task using either "novel" objects they had never encountered (New Set condition) or "novel" objects from the standard object sets that were encountered throughout testing (Standard Set condition). If scores declined on the mDNMS task over time due to the objects becoming increasingly familiar, then we predicted rats would have higher accuracy scores on the trials with new objects than on trials using the standard object sets.

[14] This range of potential object re-occurrences reflects the fact that there were three sets of objects, each with 32 object pairs. Thus, an object could re-appear a minimum 64 or maximum 96 trials later.

Materials and Method

Subjects

The same rats from the Chapter 3 experiment were used in the present experiment. The subjects were seven male Long-Evans rats (Charles River, Kingston, ON), weighing 500-575 g at the start of testing (~22 weeks old). The rats were pair-housed in polypropylene cages (48 × 25 × 20 cm) in a colony room maintained under a reverse 12:12 light-dark cycle, with light onset at 8:00 p.m. The rats had continuous access to water and each received a daily ration of ~25 g of rat chow (Charles River Rodent Animal Diet, no. 5075) in the late afternoon, after behavioral testing was finished for the day. Prior to this experiment the rats had received an average of 90 sessions on the mDNMS task using various delays (80 s, 72 hr, 3 weeks, and ~45 weeks). All procedures were approved by the Concordia University Animal Care and Use Committee, and were in accordance with the guidelines of the Canadian Council on Animal Care.

Apparatus

Behavioural testing was performed in a large multi-level environment (152 × 145 × 86 cm). The apparatus was a modified, freestanding steel cage rack, enclosed on three sides by wire mesh, with a removable, clear acrylic front panel (see Figure 2.1). The apparatus had five levels, each covered with woodchip. The top four levels were divided into two equal halves by a plastic barrier wall, and the bottom level remained undivided. A loading cage (58 × 37 × 20 cm) was placed on the top left side of the apparatus. A rat entered the apparatus via a hole in the bottom of the loading cage that was placed over a passageway leading to the top level of the apparatus. Rats traversed the different levels via wire mesh passageways located on both sides of the apparatus. The design of the apparatus was such that a rat had to climb down the passageways on the left side of the apparatus in order to gain access to the right side, which it then could ascend from level to level. The top four divided levels contained plastic rectangular platforms (30 × 12 × 1 cm) each with a recessed food well (2 cm in depth), over which stimulus objects could be placed. One platform was placed on each level on the left side of the apparatus, and on the right side of the apparatus, two platforms were placed on each level with the food wells 9 cm apart. All platforms were positioned near the middle barrier wall, in line with the passageway that provided access to the level. The room contained dim lights (40 lx) and a video camera was positioned in front of the apparatus in order for the experimenter to watch the session on a TV monitor in an adjacent room.

Stimulus objects. A total of 80 different objects were used as test stimuli. Sixty of the objects had been previously encountered on the mDNMS task ~5 times, and the last time the rats had encountered the particular objects was ~4 weeks earlier. The remaining 20 objects had never been encountered before. These 20 objects served as the novel objects on test for the New Set condition. Objects were made of plastic, metal, glass, or glazed ceramic, and ranged in size from 4 to 18 cm in height, and 4 to 13 cm in width. Each object was large enough to cover the food well but light enough to be easily displaced by a rat. There were two copies of each sample object –one for the learning phase and one for the test—and one copy of the novel object.

Behavioural procedures

The rats had been previously trained on the mDNMS task. For details on the habituation and training refer to Section 3.2.4.2. in Chapter 3.

Rats received five sessions using "novel" objects from the standard mDNMS task object sets (Standard Set condition) and five sessions using novel objects that the rats had never encountered (New Set condition). The novel objects in the New Set were paired with sample objects that had been used throughout testing. A session consisted of a sample and test phase. On the sample phase, a rat descended the apparatus encountering four distinct sample objects –one on each of the divided levels (see Figure 2.1d). One Cheerio was buried in the food well under each sample object. During the test phase, a copy of each sample object was presented next to a novel object. A Cheerio was buried under the novel object on each test level. Each session consisted of four trials, as there were four distinct sample objects in the apparatus. On the test, half of the rats encountered the sample objects paired with "novel" objects from the New Set and the remaining half encountered "novel" objects from the Standard Set. Different sample and novel objects were used each day, but on the New Set condition, the same objects served as the novel for all rats on respective sessions (i.e., all rats encountered the same four novel objects on New Set Session 1, 2, 3, 4, and 5). Rats received one session per day over the course of ten days, and the session type (Standard Set or New Set) alternated each day such that on one day a rat had a Standard Set session and on the next day it received a New Set session, and so on. The session type was counterbalanced across rats, such that approximately half the rats started with a Standard Set session and the remaining rats started with a New Set session. Consequently, this meant that both types of sessions were administered on each day of testing. Accordingly, on a particular day of testing, all of the rats encountered the same four sample objects.

The sample and novel object on each trial were paired based on similarities in size, weight, complexity, and material. The location of the novel object on each test was counterbalanced in a pseudorandom order, but within one day of testing it appeared in the same location for all rats. Between each rat, the woodchip on every level was redistributed to spread any potential odor cues left by a previous rat and each object and platform was cleaned using a 70% ethanol solution. The dependent measure was the mean percentage of correct choices on the test. A correct choice on the test was defined as the rat either displacing the novel object before displacing the sample object, or only displacing the novel object. An incorrect choice was defined as the rat only displacing the sample object, or displacing the sample object before the novel object. The mean delay between the sample and test phase for the Standard Set condition was 84 s (s = 83.01, min. = 19.8 and max. = 264.4 s) and for the New Set condition was 127 s (s = 88.37, min. = 35.8 and max. = 272.4 s).

Results

A dependent samples t-test revealed that there was no significant difference in the amount of time that rats spent investigating objects during the sample phase on the Standard Set condition and New Set condition ($t_{(6)} = 0.00$, $p = 1.00$, Hedge's $g = 0.00$).

Figure B.1 depicts rats' performance on the mDNMS task for each condition. On the Standard Set condition, the mean accuracy was 64.29% (s = 6.07) and on the New Set condition the mean accuracy was 78.57% (s = 5.56). Accuracy scores were significantly above chance on both the Standard Set condition ($t_{(6)} = 6.22$, $p = .001$, Hedge's $g = 3.33$, 95% CI [1.64, 5.02]) and New Set condition ($t_{(6)} = 13.59$, $p < .001$, Hedge's $g = 7.27$, 95% CI [4.18, 10.36]). A dependent-samples t-test revealed that scores on the New Set condition were statistically significantly higher than scores on the Standard Set condition ($t_{(6)} = 7.07$, $p < .001$, Hedge's $g = 2.45$, 95% CI [1.02, 3.88]).

Discussion

We compared rats' performance on the mDNMS task when using either trial-unique "novel" objects or "novel" objects that rats had briefly encountered before (~5 times) over widely distributed points in time (~4 weeks). Accuracy scores were significantly higher on sessions when rats made a choice between a familiar object and one they had never encountered before (New Set) compared to ones that they had previously encountered, the last time being ~4 weeks earlier (Standard Set). Moreover, the rats' scores on the New Set condition were similar to

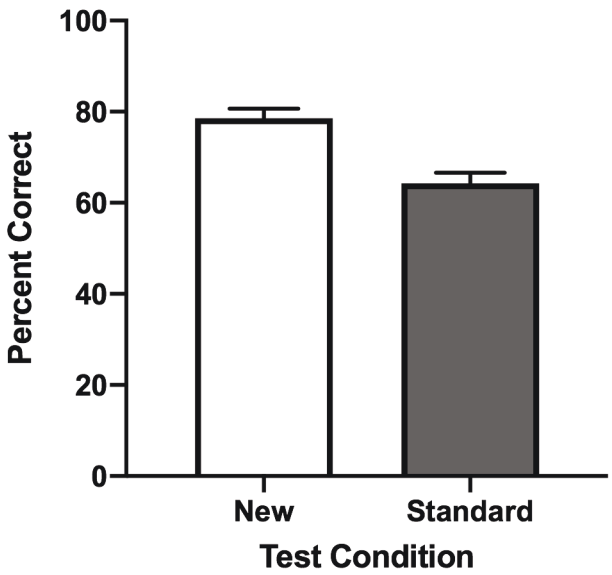

Figure B.1. Mean scores (± SEM) on the New Set condition and Standard Set condition sessions

the scores they obtained on the last five criterion sessions during mDNMS training (81%; refer to Chapter 3 results Section 3.3.2.2.). These findings suggest that using truly trial-unique stimuli, rather than pseudo-trial-unique stimuli, enhances rats' ability to discriminate between sample and "novel" objects on the test. Moreover, these findings suggest that the decline in rats' scores on the mDNMS task over time was influenced by the amount of prior exposures they had with the object sets.

There are a number of possible explanations for why using recurring objects disrupted mDNMS performance. One possible explanation is that after several exposures to an object it became so familiar that the rats had difficulty applying the nonmatching rule on the test for the sample object and a familiar "novel" object. This would suggest that the test became a measure of *recency memory*, not *recognition memory*. This also suggests that the rats had difficulty discriminating between a sample object encountered minutes earlier and a "novel" object encountered 16-24 sessions earlier (~4 weeks earlier). If the latter were true, then the amount of object exposure these rats received over cumulative mDNMS sessions (Chapter 3) was enough for a representation of these objects to be stored in long-term memory. Consequently, the persistence in memory for the objects adversely affected the rats' ability to determine which one was the *sample* on the current trial. With this reasoning, the pool of objects used on the mDNMS task needs to be even larger compared to conventional DNMS tasks to reduce the frequency for which a particular object recurs on the test. This would ensure that the objects do not become overly familiar with continued testing.

Another possible interpretation for the findings in the present experiment is that the repetition of objects over time produced *proactive interference* on the test—previously encountered objects interfered with memory at a later time (Wright, 2006, p. 166). In this case, it would mean that the rats had difficulty distinguishing between which sample object they encountered on the current trial compared to previous trials. To test the effects of proactive interference, researchers have used a *delayed same/different* task using stimuli (e.g., pictures) presented on a touch screen. On the task, the subject is presented with a sample stimulus, followed by a delay. On the test, either the *same* or a *different* stimulus is presented on the screen next to a white square. When the same stimulus is presented, the subject gets a reward if it touches the same stimulus, and when a different stimulus is presented the subject gets a reward if it touches the white square next to the stimulus. Different sample stimuli are used on each trial,

but to assess proactive interference, some of the "different" test trials are manipulated such that a sample stimulus from the preceding trial is presented as the test stimulus on the current trial. Proactive interference could make it difficult for the subject to determine whether the test stimulus matches the sample stimulus on the current trial or the sample from the previous trial. Compared to "different" trials with no proactive interference, both pigeons (Wright, Katz, & Ma, 2012) and nonhuman primates (Devkar & Wright, 2016) score significantly lower, indicating a tendency to incorrectly identify the test stimulus as the sample stimulus on the current trial. Moreover, the effects of proactive interference on performance decline by increasing the number of trials between the presentation of the sample stimulus and its reappearance as a test stimulus (Devkar & Wright, 2016; Wright et al., 2012; Wright, Kelly, & Katz, 2018). The build up of proactive interference has typically been examined for stimuli encountered *within* a test session, not across sessions occurring over days. If the results from the present experiment do in fact reflect a build up of proactive interference, then it suggests that these intrusions can occur for information encountered over long periods of time between object presentations (e.g., dozens of trials and days earlier). If this were the case, then it suggests that rats' long-term memory is grossly underestimated. Accordingly, using a large object set to reduce the recurrence of objects would be needed to eliminate the potential for proactive interference to disrupt performance over time.

We can rule out the possibility that the differences in accuracy scores on the Standard Set and New Set condition was due to differences in the amount of time rats spent investigating objects on the sample phase during each condition, as rats spent a similar amount of time investigating the sample objects during both conditions. Moreover, the results from the probe tests from the experiment described in Chapter 3 revealed that these rats were not relying on olfactory cues to successfully locate the reward on the test. Regardless, if rats were in fact relying on olfactory cues to locate the food reward, then we would not expect to find a significant increase in accuracy scores on the New Set condition compared to the Standard Set condition. Lastly, it is unlikely that the features of the novel objects in the New Set evoked an *a priori* preference relative to the sample objects as they were matched based on similarities in size, material, and complexity (e.g., a New Set novel object that was made of smooth plastic was paired with a sample with the same features).

The rats in the present experiment were 18 months old at the onset of testing. Thus, it is

possible that the differences in performance on the New Set and Standard Set conditions reflects age-related deficits in working-memory abilities, rather than difficulties distinguishing between two familiar objects on the test. On the New Set condition, only the "novel" objects were new, whereas the sample objects used on each session had been encountered before. Accordingly, this raises the possibility that on the New Set condition the rats could have successfully solved the tests by relying on long-term memory for the sample objects (i.e., which object do you have a stored representation of in long-term memory) and not working-memory (i.e., which object did you previously encounter on this particular session). Conversely, on the Standard Set condition tests, rats could only rely on memory for object information learned on that particular session to successfully discriminate between the novel and sample on the test. One way to rule out this alternative explanation would be to use an entirely new set of objects to serve as both sample and novel objects on the New Set condition.

Overall, the findings from this experiment revealed that rats' performance improved on the mDNMS task when using trial-unique novel objects compared to objects that recurred over time. The length and number of object encounters was enough to interfere with accuracy on the test when discriminating between a recently encountered object and one encountered weeks earlier. Thus, these findings reveal that rats are capable of remembering objects over long periods of time when provided with several brief encounters over widely distributed points in time. Accordingly, a large object set with few recurring objects is required when administering many trials on the mDNMS task because using recurring objects disrupts performance on the task.