MODULATORY MECHANISMS OF OBJECT-RECOGNITION MEMORY IN THE PERIRHINAL CORTEX

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ABSTRACT

Modulatory mechanisms of object-recognition memory in the perirhinal cortex

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The present thesis examined whether acetylcholine (ACh) and 17-β estradiol (E2) modulate object-recognition memory (ORM) and perirhinal cortex (PRh) function. ORM was assessed using the Novel-Object Preference (NOP) test or the delayed non-match-to-sample (DNMS) task. The first goal was to investigate whether acetylcholine (ACh) acts via muscarinic receptors (mAChR) in the PRh to modulate novelty preference and novelty-related neuronal activation. Male rats received intra-PRh infusions of a mAChR antagonist or vehicle in the PRh before or after the familiarization phase of the NOP test, then tested 4- or 24-hr later. These infusions were also given before novel- or familiar-object exploration. The antagonist prevented novelty preference regardless of retention delay and timing of infusion. While novel-object exploration resulted in increased PRh activation, this increase was prevented by mAChR antagonism. These results are consistent with the idea that ACh (via mAChR in the PRh) modulates novelty preference and neuronal activation following novel-object exploration.

A second goal was to determine whether intra-PRh modulates ORM and PRh-mediated synaptic plasticity in ovariectomized rats. Intra-PRh infusions of E2 or vehicle were given immediately before, immediately after, or two hours following the familiarization phase of the NOP test. Intra-PRh infusions of E2 or

vehicle were also given before the DNMS task. Enhanced novelty preference was observed on a 72-hr retention test when E2 was administered immediately before or after familiarization. Intra-PRh E2 reduced accuracy scores on the DNMS task following a 3-min retention delay. A subsequent study examined whether E2 modulates ORM via estrogen receptor beta (ERβ). Intra-PRh infusions of a selective ERβ agonist enhanced novelty preference following a 4- and 72-hr retention delay, but had no effect on accuracy on the DNMS task. The final study compared synaptic density of PRh neurons following proestrous and estrous, and following high E2 replacement or no replacement in ovariectomized rats. High levels of E2 were associated with reduced synaptic density. These results support the idea that while E2 (via ERβ) enhances novelty preference, it impairs ORM (independent of ERβ). The modulatory effect of E2 on synaptic density in the PRh is a potential mechanism through which E2 influences ORM.

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CONTRIBUTION OF AUTHORS

1. **Muscarinic receptors in the perirhinal cortex influence novelty preference and neuronal activation (Chapter 2)**

   The study was designed by Nicole Gervais, with the assistance of Dr. Dave Mumby. Nicole conducted all procedures related to the experiment, and wrote the chapter with the assistance of Dr. Mumby and Dr. Wayne Brake. Dr. Barbara Woodside provided laboratory resources and guidance for Fos analysis.

2. **Systemic and intra-rhinal-cortical 17-β estradiol administration modulate object-recognition memory in ovariectomized female rats (Chapter 3)**

   The study was designed by Nicole Gervais with the assistance of Dr. Mumby and Dr. Brake. Nicole conducted all procedures related to the experiments, and wrote the manuscript for publication, with the assistance of Dr. Mumby and Dr. Brake. Sofia Jacob assisted with data collection.

3. **Intra-perirhinal estrogen receptor beta and object-recognition memory in ovariectomized rats (Chapter 4)**

   The study was designed by Nicole Gervais with the assistance of Dr. Mumby and Dr. Brake. Nicole conducted all procedures related to the experiment, and wrote the chapter with the assistance of Dr. Mumby and Dr. Brake.

4. **Attenuation of synaptic density in the perirhinal cortex following 17-β estradiol replacement in the rat (Chapter 5)**

   The study was designed by Nicole Gervais with the assistance of Dr. Brake. Nicole conducted all procedures related to the experiment, and wrote the chapter with the assistance of Dr. Mumby and Dr. Brake. Dr. Mumby provided laboratory resources.

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LIST OF ABBREVIATIONS

A	Anterior
a35	Broadmann's area 35
a36	Broadmann's area 36
ACh	Acetylcholine
aCSF	artificial cerebrospinal fluid
AD	Alzheimer's disease
AIP	Agranular insular cortex
AMPAR	alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor
AMY	Amygdala
ANOVA	Analysis of variance
CamKII	Calcium/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CREB	cyclic AMP response element binding protein
D	Dorsal
DNMS	Delayed non-match-to-sample
DPN	Diarylpropionitrile
E2	17-β estradiol
EB	Estradiol benzoate
EC	Entorhinal cortex
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
GAMA	Gamma-aminobutyric acid
Glu	Glutamate
GPER1	G protein-coupled estrogen receptor 1
HPC	Hippocampus
IEG	Immediate early gene
i.p.	Intraperitoneal injection
IR	Immunoreactive
L	Lateral
LTP	Long-term potentiation
LTD	Long-term depression
M	Medial
mAChR	Muscarinic receptor
MAPK	Mitogen-activated protein kinase
mPFC	Medial prefrontal cortex
MTL	Medial temporal lobe
NMDAR	n-methyl-d-aspartate receptor
NOP	Novel-object preference
ODN	Oligodeoxynucleotide
ORM	Object-recognition memory
OVX	Ovariectomized

P	Posterior
PHC	Parahippocampus cortex
PIR	Piriform cortex
PKA	Protein kinase A
POR	Postrhinal cortex
PRh	Perirhinal cortex
PPT	Propyl pyrazole triol
SEM	Standard error of the mean
s.c.	Subcutaneous injection
SD	Standard deviation
TeA	Temporal association cortex
V	Ventral

CHAPTER 1

General Introduction

The ability to learn and remember is an important aspect of survival. Many of the learning and memory abilities seen in humans are also observed in animals, including recognition memory. Recognition of objects (i.e. object-recognition memory; ORM), which is the ability to discriminate the familiarity of objects previously encountered (Aggleton & Brown, 1999), is demonstrated in both non-human primates and rodents. Studies using these species allow for investigations into the role of specific brain regions, the contributions of modulators to ORM within these regions, and the identification of neurobiological correlates of memory formation. These studies have led to the discovery that the perirhinal cortex (PRh), which is present in all primates and rodents, plays an important role in ORM (Winters, Saksida, & Bussey, 2008). However, the modulators and mechanisms within the PRh that contribute to ORM remain unclear. The main purpose of this thesis is to investigate whether acetylcholine and 17-β estradiol influence ORM through actions in the PRh, and whether their actions within this region affect neuronal correlates of learning and memory.

In primates, the PRh is located in the medial temporal lobe (MTL). In addition to the PRh, the other MTL structures include the hippocampus (HPC), amygdala (AMY), entorhinal cortex (EC), and parahippocampal gyrus (rodents have an analogous structure called the postrhinal cortex). Interest in the MTL grew in the 1950s following observations that surgical removal of this structure produced severe memory deficits. Specifically, Henry Molaison (previously known as patient H.M.) received bilateral removal of the MTL as treatment for his severe epilepsy. Following his surgery, Scoville and Milner (1957) reported that

Henry presented with severe memory impairments. This included an inability to recognize individuals he met following removal of his MTL. This observation lead to the development of amnesia models in macaques to investigate the contribution of the individual MTL structures to different learning and memory abilities. To model the amnesia seen in Henry and other patients with MTL damage, bilateral lesions were surgically produced in the MTL of macaques and performance on ORM tests was measured. The ORM test used by researchers is called the delayed non-match-to-sample (DNMS) task (Mishkin, 1978). The macaque is initially shown an object (i.e. sample) and following a delay, the sample object is presented along with a novel one. A different set of objects are used on each trial. The macaque is trained to displace the novel object in order to obtain a food reward. Reliably displacing the novel object involves recognizing the sample object as familiar, which is why this task is considered an ORM test. It was not until the late 1980s that this memory ability was assessed in rodents.

To identify modulators of learning and memory, research has focused on the role of specific neurotransmitters, including acetylcholine and dopamine. Acetylcholine (ACh) has been extensively studied in the HPC and is known to influence the acquisition of spatial information, for which the HPC plays an important role. ACh likely affects the functions of other MTL structures, including the PRh, where cholingergic afferents from the medial septum terminate (Winters & Bussey, 2005a). However, little research has been done on the effects of ACh on functions of the PRh.

Other than neurotransmitters, stress and gonadal hormones (including estrogens) also modulate learning and memory abilities. Most of the research on the effects of estrogens on cognition in rodents focus on abilities that involve the HPC, with little work into their role in other MTL structures. One goal of this thesis is to examine the contribution of both ACh and 17-β estradiol (E2) in the PRh on ORM. Another aim is to define how these agents affect neuronal correlates of ORM, including PRh activity following object exploration, and synaptic density in the PRh.

The following sections describe the importance of the PRh in ORM. The discussion begins with a review of the location, boundaries, and connectivity of the PRh (Section 1.1), then moves to a discussion on visual recognition memory in humans (Section 1.2), and ORM in rhesus macaques (Section 1.3), and rats (Section 1.4). The focus shifts to a discussion of mechanisms of ORM (Section 1.5). A review of neurotransmission and actions of hormones (including E2) in the PRh is given in Section 1.6. Section 1.7 summarizes the literature review and provides the rationale for the thesis (Section 1.7), and Section 1.8 describes the main hypotheses addressed in subsequent chapters.

1.1 Properties of the Perirhinal Cortex (PRh)

1.1.1 Anatomy of the PRh

In primates, the PRh is located in the MTL, bordered by both the EC (medially) and parahippocampal gyrus (posteriorly) and the rhinal sulcus (ventrally). The anterior, but not posterior HPC is located dorsal to the PRh (Bear, Connors, & Paradiso, 2007; Kandel, Schwartz, & Jessel, 2000). Although

the brains of rats do not contain a temporal lobe, they do include the same structures of the primate MTL, with the exception of the parahippocampal gyrus, which has an analogous structure in the rat (i.e. postrhinal cortex). *Figure 1.1* presents schematic representations of the rat, macaque, and human brain, and includes the location of the PRh and adjacent structures. In the rat, the PRh is bordered rostrally by the posterior agranular insular cortex (AIP), caudally by postrhinal cortex (POR), laterally by the rhinal sulcus, dorsally by the temporal association cortex (TeA) and ventrally by the lateral EC (Burwell, 2001; Kealy & Commins, 2011). The PRh is comprised of Broadmann's area 35 (a35) and 36 (a36; Broadmann, 1909), although some define the PRh as a35 alone (i.e. Paxinos & Watson, 1998). Area 35 begins slightly more rostrally than a36, beginning approximately 2.45-2.80 mm posterior to Bregma (Burwell, 2001). Area 35 is situated along the ventral bank of the rhinal sulcus and a36 along the dorsal bank (Burwell, 2001; Kealy & Commins, 2011). Both areas continue until approximately 6.72 mm from Bregma (Burwell, 2001). Although there is some debate as to whether the PRh and postrhinal cortices are distinct structures (Kealy & Commins, 2011), for the present thesis, the classification described by Burwell (2001) will be used. So the PRh will be considered as a separate structure from the postrhinal cortex. *Figure 1.2* presents an illustration of the rat brain, depicting the location of a35 and a36, as well as surrounding brain structures.

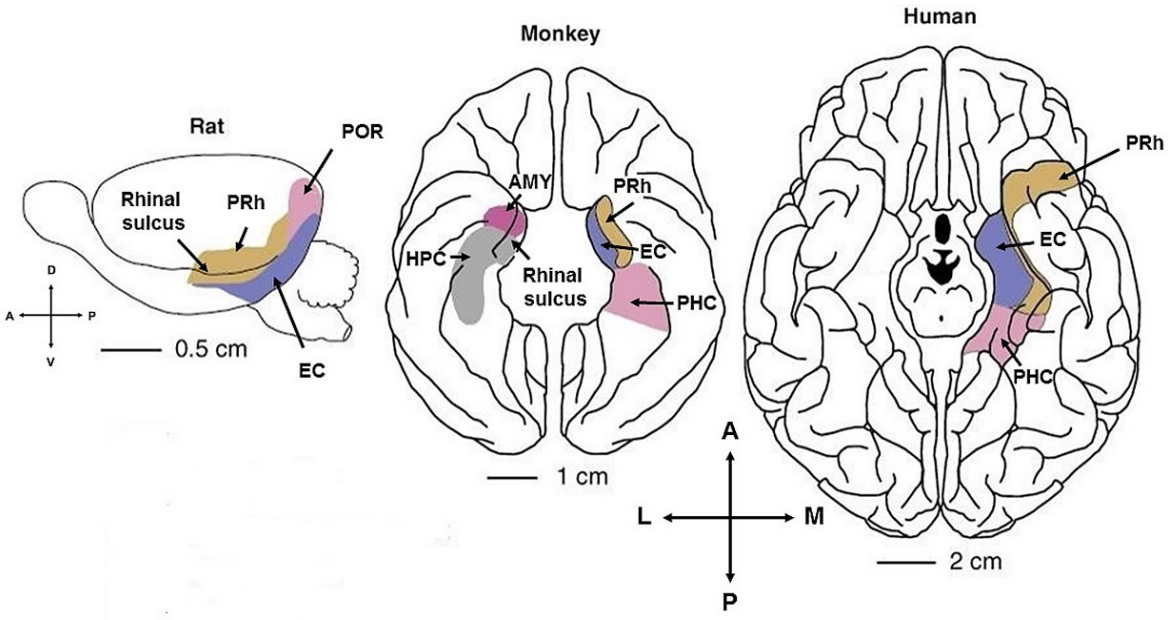

Figure 1.1. Illustration of lateral view of rat brain (left) and ventral view of a rhesus macaque (middle) and human (right) brain. Location of the PRh and adjacent regions are depicted. This image is adapted from Murray, Bussey, and Saksida (2007). *Note*: A = Anterior; AMY = Amygdala; D = Dorsal; EC = Entorhinal cortex; HPC = Hippocampus; L = Lateral; M = Medial; P = Posterior; PHC = Parahippocampus cortex; POR = Postrhinal cortex; PRh = Perirhinal cortex; V = Ventral.

One cytoarchitectonic property of the PRh that distinguishes it from neighbouring cortical regions is the complete absence of layer IV in a35 and a poorly defined layer IV in a36 (Burwell, Witter, & Amaral, 1995). The other layers (I-III and V-VI) are present in both a35 and a36. However, there are cytoarchitectonic differences between the two sub-regions. For one, layer I of a35 tends to be thicker than a36, and the pyramidal neurons in layer V of a35 tend to have a heart-shape. In addition, there is a general radial organization of the cells in all layers of a35 that is not observed in a36. Layers II and III are distinct from each other in a36, but are not in a35 (Burwell, 2001; Burwell & Amaral, 1998a,b; Krieg, 1946).

The PRh receives projections from unimodal and polymodal association areas. Unimodal association areas integrate afferent information for a single sensory modality, then project to polymodal association areas, which integrate afferent information from multiple sensory modalities (Kandel, Schwartz, & Jessell, 2000). Area 35 receives substantial input from the piriform cortex (PIR), a unimodal association area (a36 also receives PIR input, although much less). Area 36 receives the majority of remaining input from unimodal association cortices (auditory, somatosensory, visuospatial, visual, and gustatory; Burwell, 2000).

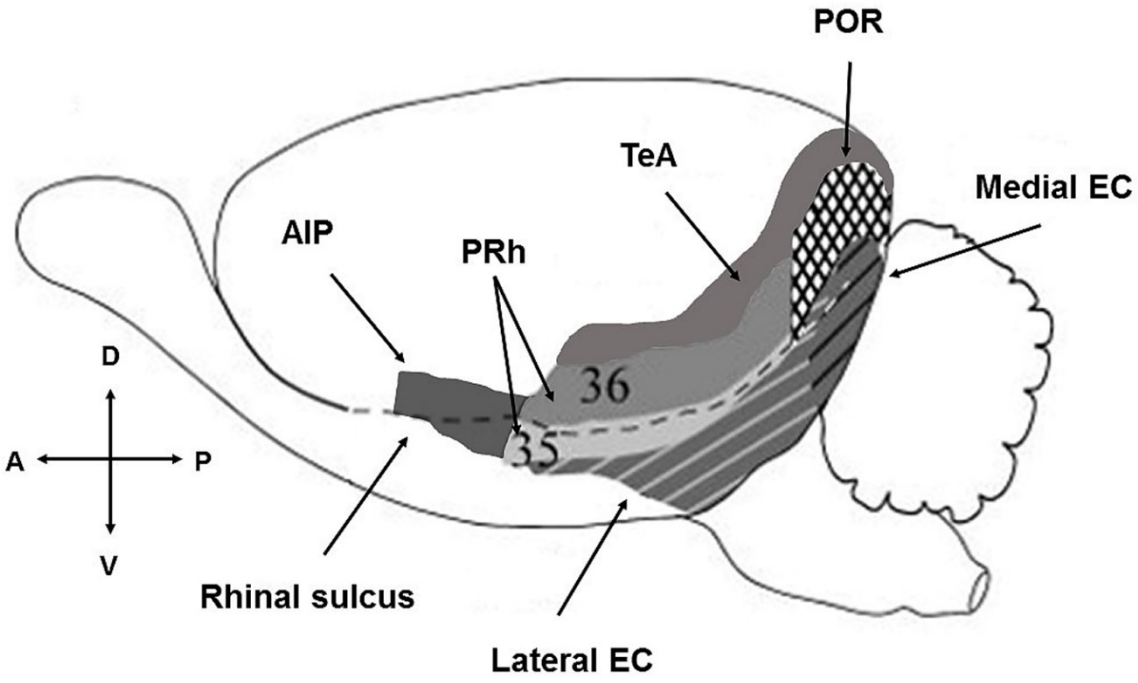

Figure 1.2. Illustration of lateral view of rat brain showing location of a35 and a36 of PRh. Also shown are the areas bordering the PRh. This figure is adapted from Burwell (2001). *Note*: 35 = Broadmann's area 35; 36 = Broadmann's area 36; A = Anterior; AIP = agranular insular cortex – posterior portion, D = Dorsal; EC = Entorhinal cortex; P = Posterior; POR = Postrhinal cortex; PRh = Perirhinal cortex; TeA = Temporal association cortex; V = Ventral.

Although the PRh receives input from all unimodal association cortices, the majority of the input is olfactory. The polymodal association areas that project to the PRh include the ventral TeA, agranular insular, orbital frontal, posterior parietal, and medial frontal, with the majority of polymodal afferents originating from the ventral TeA (Burwell, 2000). Consequently, the PRh receives input from all sensory modalities. Other afferents to the PRh include input from the ventral HPC (from CA1 and subiculum, predominantly to a35), parasubiculum (a35), EC, postrhinal cortex (predominantly a36), AMY, striatum, and thalamus (predominantly area 36; Agster & Burwell, 2013; Burwell et al., 1995; Furtak,Wei, Agster, & Burwell, 2007).

In addition to all the sensory input to the PRh, there is also output from the PRh to the same cortical regions, including the PIR, insular (originating mainly in a35), frontal, temporal, and parietal cortices (mainly from a36; Agster & Burwell, 2009). The projections to the PIR, frontal and temporal regions are stong, with weaker projections terminating in the cingulate and occipital regions. In general, it appears as though there is considerable reciprocity between the PRh and cortical regions (Agster & Burwell, 2009). In addition to these cortical regions, the PRh also sends strong projections to the lateral EC (LEC, mainly from a35; Burwell & Amaral, 1998a) and weak projections to the CA1 and subiculum of the vHPC (Agster & Burwell, 2013). The PRh provides input to the vHPC both directly and indirectly via the LEC (Agster & Burwell, 2013). Other important projections from the PRh include the AMY and basal ganglia (Furtak, Wei, et al., 2007).

1.1.2 Properties of neurons within the PRh

Pyramidal neurons are the most common cell type in the PRh, accounting for 49% of all neurons in this structure (Furtak, Russell, & Brown, 2007). However, they are less abundant than in other cortical areas (Kealy & Commins, 2011). In the PRh, they are morphologically divisible into one of 5 categories: 1) upright (most common), 2) horizontal, 3) inverted, 4) bifurcating, and 5) oblique (Kealy & Commins, 2011). The majority of pyramidal neurons are in layers II/III and V.

The PRh has been characterized as a “slow cortex”, because of low myelin content of its neurons, which slows the propagation of the current down the axon. In addition, there are PRh neurons with different spiking patterns, or patterns of action potentials. The most common are either “regular spiking” (predominate in layer V) or “late spiking” neurons (predominate in layers II/III and VI; Furtak, Russell et al., 2007; Kealy & Commins, 2011). Both long-term potentiation (LTP) and long-term depression (LTD) can be induced in the PRh, although the latter is more commonly associated with PRh-mediated learning and memory (Kealy & Commins, 2011).

PRh neurons are capable of Glu neurotransmission as they express all three types of ionotropic Glu receptors, N-methyl-D-aspartate receptor (NMDAR), alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR), and kainate receptor, and the metabotropic receptors. The abundance of these receptors, particularly the ionotropic Glu receptors, is lower than in other cortical areas (Kealy & Commins, 2011) and their distribution is layer specific. Both

NMDAR and AMPAR density is higher in layers I-III, whereas the density of kainite receptors is higher in layers V-VI (Kealy & Commins, 2011). PRh neurons receive cholinergic innervation via the medial septum (Winters & Bussey, 2005a), and express both nicotinic and muscarinic receptors (Kealy & Commins, 2011). The PRh is also capable of neurotransmission via dopamine, as it expresses, D1 (D1R), dopamine D2 (D2R), and dopamine D4 (D4R) receptors, serotonin (via 5-HT_{1A} & 5-HT_2 receptors), epinephrine (as it expresses α_2-adrenergic receptors and norepinephrine transporters) and to a lesser extent GABA (as $GABA_b$ receptors are expressed and weak GABAergic innervation is received from the temporal cortex and EC; Kealy & Commins, 2011). Along with neurotransmitters, there is evidence for signaling via neurotrophins (including nerve growth factor and brain-derived neurotrophic factor; Kealy & Commins, 2011) as well as steroid hormones (i.e. estrogens; Blurton-Jones & Tuszynski, 2002).

1.1.3 Functional implications of PRh properties for learning and memory

As described in Section 1.1.1, the PRh shares reciprocal connections with uni- and polymodal sensory regions, including the PIR and TeA. The PRh also receives reciprocal projections from the vHPC and LEC. Given the known role of the HPC in learning and memory, these reciprocal connections suggest a role of the PRh in information processing. Both LTP and LTD, which are proposed synaptic mechanisms of long-term memory (Brown & Bashir, 2002; Kandel, 2001) can be induced in PRh.

The following sections review research in humans, macaques, and rodents that support a role of the PRh in one learning and memory ability, namely recognition memory.

1.2 Visual recognition memory in humans

Visual recognition memory in humans is thought to involve the PRh (Hodges & Graham, 1998; Schmolck, Kensinger, Corkin, & Squire, 2002). This conclusion is based on studies assessing impairments observed in patients that experienced damage to the temporal neocortex, which includes the PRh, but spares the HPC. Patients in these studies presented with impaired visual recognition memory, but intact episodic memory (i.e. ability to recall autobiographical details and events; Hodges & Graham, 1998; Schmolck, Kensinger, Corkin, & Squire, 2002). Lesions observed in these patients were not restricted to the PRh, which limits conclusions that can be drawn about the contributions of damage in this structure to specific aspects of the memory impairment. Therefore, the reported impairments cannot be attributed to the PRh alone.

The brain damage experienced by Henry Molaison and other patients with damage to the MTL was also not restricted to one structure. Although the neurosurgeons who lesioned Henry's brain were originally targeting the HPC, the surgical tools available at that time made it difficult to limit the extent of damage. The extent of the extrahippocampal damage created by the surgery was not initially known and so the memory impairments that Henry presented with post-surgery were erroneously attributed to the HPC damage. However, magnetic

resonance imaging was later used on Henry (as it only became available decades later) to observe the extent of damage to his MTL. Damage to the HPC was confirmed, although it was discovered that much of the structure was left intact. In addition to HPC damage, extensive damage to the PRh, EC, parahippocampal gyrus and AMY occurred (Corkin, Amaral, Gonzalez, Johnson, & Hyman, 1997). Although this discovery was an important one, as it suggests the impairments observed in Henry (which included visual recognition memory) are not attributable to HPC damage alone, many researchers at that time continued to argue that the HPC alone is required for visual recognition memory.

The absence of information regarding memory ability prior to brain damage also limits the extent to which conclusions can be drawn about the contribution of specific structures to visual recognition memory. Memory tests administered following damage can inform researchers about whether an ability is impaired or not, but does not indicate whether the brain damage is responsible for the impairments. Studies involving animal models of specific memory abilities are a useful adjunct to research on human MTL amnesia, as pre-surgery memory ability can be assessed. Indeed, animal models of human MTL amnesia provided the first demonstration that the PRh is important for visual recognition memory, which was confirmed in subsequent studies.

1.3 ORM in rhesus macaques

The first animal models of human MTL amnesia used non-human primates, particularly rhesus macaques, to assess the functional implications of damage to that region. This research allowed for more control over the location

and extent of lesions, particularly as techniques were refined over the decades that followed the initial discovery of amnesia resulting from MTL damage in humans. In addition, research with macaques allowed for both pre- and post-damage assessment of memory ability.

Object-recognition memory (ORM), which is defined as the ability to discriminate the familiarity of objects previously encountered (Aggleton & Brown, 1999), is observed in macaques and resembles visual recognition memory in humans. The delayed non-match-to-sample (DNMS) task, which shares key features with visual recognition memory tests used in humans (Mumby, 2001), was designed to assess this ability in macaques across different retention delays. The task involves presenting a macaque with an object. This object is placed over a well containing a food reward. The macaque is trained to displace this object in order to retrieve the food. Once it has done so, a screen is placed in front of the macaque's cage and the object is removed and replaced with a copy of itself (referred to as the sample object) and a novel object. This time, only the novel object is paired with reward. Once the screen is removed a few seconds later, the macaque must displace the novel object in order to obtain the reward. A different object pair is used on each trial. Reliabily displacing the novel object across several trials requires recognizing the sample object. Macaques have been shown to reliably displace the novel object with a high-level of accuracy (90 of 100 trials correct), and this accuracy is maintained when retention delays are as long as 120 s (Mishkin, 1978).

To investigate the role of the MTL in ORM, Mishkin (1978) first trained macaques on the DNMS task until they achieved a 90% performance criterion, after which they received either bilateral lesions to the HPC alone, AMY alone, or combined HPC and AMY. A fourth group received no lesion and served as the control condition. Following surgery, each macaque was retrained to pre-operative performance criterion before being tested at longer retention (30, 60, and 120 s) delays. The control macaques, and those with damage to either the HPC or AMY continued to perform well at all retention delays. The only macaques to demonstrate severe impairments were those that received damage to both the HPC and AMY, as they demonstrated poor accuracy at all retention delays (30, 60, and 120 s). This was the first demonstration of amnesia produced by damage to the MTL in non-human primates. Not only did Mishkin replicate impairments observed in humans with damage to comparable brain regions, he also provided clear evidence that damage to the MTL produces amnesia as the macaques with combined lesions were able to perform the task before damage occurred. Due to the surgical techniques available at the time, damage was not restricted to the HPC and AMY, but extended to adjacent structures of the rhinal sulcus, including the PRh and EC. The importance of the rhinal cortices in ORM was not demonstrated until much later when Murray and Mishkin (1986) revealed severe impairments on the DNMS task following combined lesions to the AMY and PRh/EC, which produced comparable impairments as those seen following combined AMY and HPC lesions in the earlier study (Mishkin, 1978). Taken together, the findings from these two studies suggest that the amygdala, EC,

PRH, and parahippocampal gyrus are involved in ORM, whereas the HPC has a limited role.

Studies conducted a few years later confirmed the importance of the PRh and not the AMY, EC, or HPC in ORM. Combined damage to the PRh and parahippocampal gyrus resulted in severe performance deficits on the DNMS task, whereas damage restricted to the AMY did not (Zola-Morgan, Squire, Amaral, & Suzuki, 1989). In addition, Meunier, Bachevalier, Mishkin, and Murray (1993) reported severe performance deficits following PRh, but not EC lesions. Although deficits in DNMS task performance following HPC lesions were reported (Zola-Morgan, Squire, Clower, & Rempel, 1993), these impairments were more severe when combined with damage to the PRh (Meunier et al., 1996; Zola-Morgan et al., 1993). In addition, damage restricted to the HPC resulted in performance deficits when macaques received no pre-surgery training (Zola-Morgan & Squire, 1986). Taken together, studies using macaques provide convincing evidence that the PRh plays an important role in ORM. Although lesions to the HPC also result in impaired DNMS task performance, this occurs when no pre-surgery training is given and also when damage extends to extrahippocampal structures including the PRh.

1.4 ORM in rats

1.4.1 DNMS task. Several rodent versions of the DNMS task have been developed, including one by Aggleton (1985) that involves a Y-shaped maze. In this version of the task, a rat is placed in the start arm and is trained to make a choice between two adjacent arms. Both arms contain identical copies of the

same object. After choosing one of the arms, a door is lowered, restricting the rat to the chosen arm. The rat is rewarded for choosing an arm and is able to investigate both the object and arm, while new objects are placed in the other two arms. In one of the arms is a copy of this now familiar object and in the other, a novel object is placed. The door is then lifted, allowing the rat to leave the arm and choose between the arm that contains the familiar and the one with the novel object. During this phase of the trial, the rat is only rewarded when it enters the arm containing the novel object. Much like the macaque version of this task, delays between the familiar and choice phases of the task can be introduced, once the rat reliably chooses the arm containing the novel object.

Another version was later developed by Mumby, Pinel and Wood (1990). In this version of the task, the rat is placed in the centre of a rectangular apparatus, with doors at both ends preventing access to goal arms. At the start of each trial, one of the doors is lifted allowing the rat to approach and displace an object. Once the object is displaced, the rat receives a food reward. The rat is trained to return to the centre compartment, where the door is lowered and during this interval, the experimenter places the now familiar object in the other goal arm along with a novel object. Following a delay, the door blocking the second arm is lifted allowing the rat to enter the arm and investigate both objects. Displacing the novel object results in delivery of a food pellet. *Figure 1.3* illustrates the four-step procedure for administering one trial of the DNMS task described by Mumby and colleagues (1990). These two versions of the DNMS task, as well as others (i.e. Kesner, 1991; Rothblat & Hayes, 1987), have been

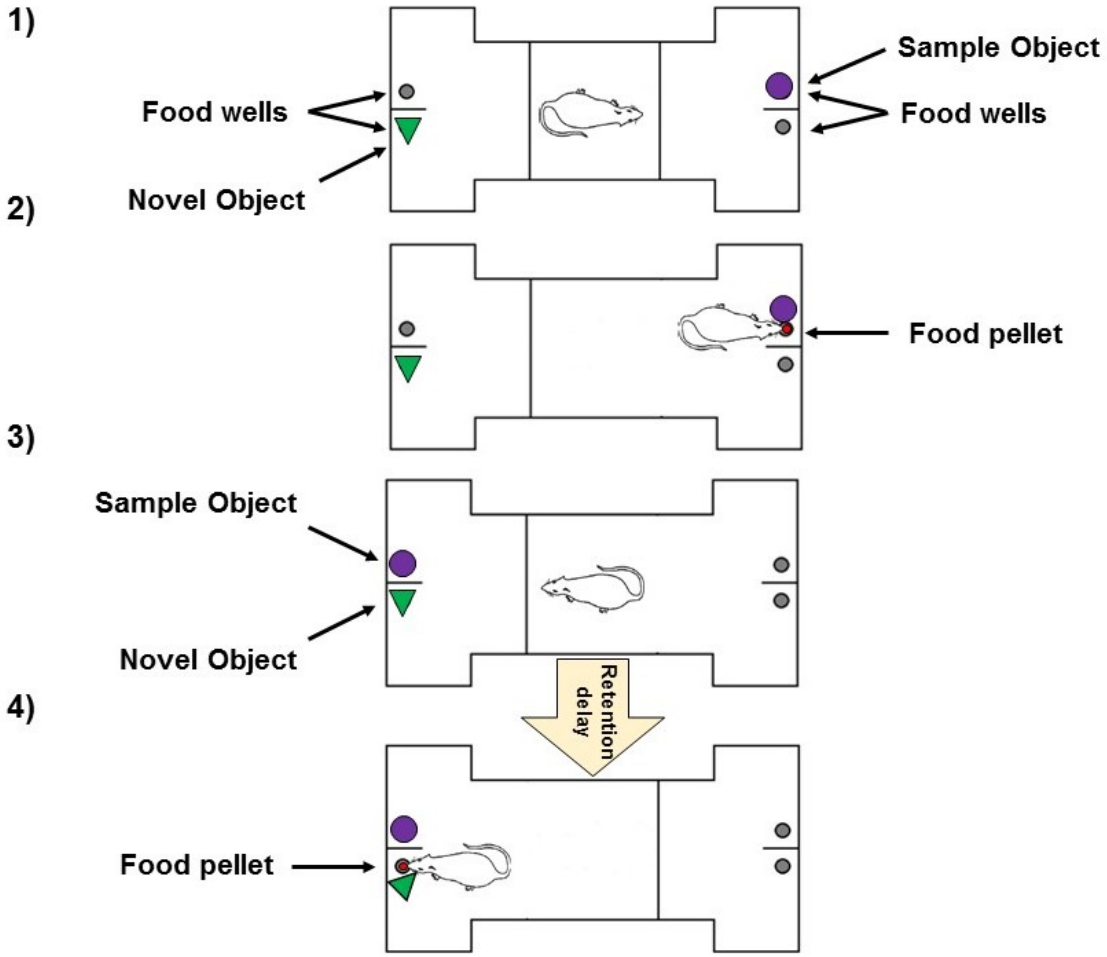

Figure 1.3. Schematic representation of the rodent version of the DNMS task used in Mumby, Pinel, and Wood (1990).

used to assess ORM in rats following selective damage to the HPC, AMY, and rhinal cortices. In general, damage restricted to the HPC or AMY results in either no impairment (Aggleton, Hunt, & Rawlins, 1986; Aggleton, Blint, & Rawlins, 1989; Rothblat & Kromer, 1991), or mild impairment (Mumby, Wood, & Pinel, 1992) on the DNMS task. Combined lesions to the HPC and AMY produced performance deficits in one study (Aggleton et al., 1989), but not another (Mumby et al., 1992). However, the combined lesions produced in the Aggleton and colleagues (1989) study included extensive damage to the PIR, which may have contributed to the observed impairment. Although the majority of studies report no performance deficits following damage to the HPC, some report severe impairments. Similar to the research using rhesus macaques, these impairments are only observable when no pre-surgery training occurs. For example, Clark, West, Zola, and Squire (2001) trained rats on the DNMS task following either HPC lesions or no lesion. Following post-surgery training on the DNMS task, rats with damage to the HPC were impaired relative to controls following longer retention delays. However, when pre-surgery training was given, DNMS task performance was unaffected by HPC damage (Aggleton et al., 1986). Although reliable performance on the DNMS task requires intact ORM, it also requires the ability to learn, remember and apply the non-matching rule (Ennaceur & Delacour, 1988). Thus, when rats fail to perform reliably on this task, they may do so because their ability to recognize the familiar object is impaired, or because they’re unable to apply the non-matching rule. The pattern of impairment across different retention delays is useful for distinguishing between these two

possibilities. Impairments that are delay-dependent, with normal performance following short delays (as was reported in Mumby & Pinel, 1994), is consistent with a deficit in ORM.

Evidence suggests that HPC damage results in poor performance on nonspatial working-memory tasks that involve a non-matching rule. For example, Olton and Feustle (1981) trained rats on a nonspatial working-memory task that involves using visual features of arms in a radial-arm maze. Rats were first trained on the task until a performance criterion was reached, then received fornix lesions, which are known to disrupt HPC functions. Following surgery, fornix-lesioned rats were no longer able to perform the task. This study and others (Jagielo, Nonneman, Isaac, & Jackson-Smith 1990; Rafaelle & Olton, 1988), confirm that HPC damage impairs nonspatial working-memory when visual features of goal arms or alleys serve as stimuli. As reviewed by Mumby (2001), later experiments conducted by Rawlins, Aggleton, and colleagues (Cassaday & Rawlins, 1995, 1997; Rawlins, Lyford, Seferiades, Deacon, & Cassaday, 1993; Yee & Rawlins, 1994**)** provide compelling evidence that disruptions to HPC function impair nonspatial working memory without affecting ORM. Specifically, these studies demonstrate that the size and complexity of the stimuli used to test nonspatial working-memory ability determines whether or not impairments are observed. Rats with HPC damage are impaired on nonspatial working memory when stimuli are large whereas no impairments are observed with smaller, more complex stimuli. The larger stimuli are thought to be more similar to places than objects whereas the opposite is thought to be true for

smaller stimuli. Taken together, these findings suggest that disrupting HPC function does not impair ORM.

In contrast to disruptions in HPC function, impairments following PRh damage are observed on the DNMS task. For example, Mumby and Pinel (1994) observed impaired DNMS task performance following damage restricted to the PRh and EC. As seen in macaques with damage to the PRh (e.g. Meunier et al., 1993; Murray & Mishkin, 1986), the impairments were delay-dependent, occurring following longer, but not short, retention delays. Delay-dependent deficits in DNMS task performance are also reported elsewhere (Wiig & Bilkey, 1995). Despite the consistency in the pattern of performance deficits observed on the DNMS task across these two studies, and across species, too few studies have directly addressed the role of this brain region to allow for conclusions to be made about its role in ORM. One likely reason for the lack of data is that training on this task is very time consuming, requiring several hundred trials before performance criterion is reached (Mumby et al., 1992, 1994). It wasn't until a different test of ORM (i.e. novel-object preference test) was introduced that more evidence emerged regarding a role of the PRh in this ability.

1.4.2 Novelty-object preference (NOP) test. In 1988, Ennaceur and Delacour described a different test of ORM that uses the rodents' spontaneous exploratory behavior. For this reason, the test was originally called the "spontaneous object recognition" (SOR) test. However, it will be referred to as the Novel-Object Preference (NOP) test in this thesis as this name more accurately reflects the behavior being investigated (i.e. novelty preference). The NOP test involves two

phases, the familiarization phase, where the rodent is presented with 1-2 identical objects in an open field arena. The rat is allowed to investigate the arena and both objects before being removed from the open field for a retention delay. Typically, the length of the familiarization is fixed (3 or 5 min) and the amount of time the rodent explores each copy of the object is scored. However, some researchers end this phase after 30 s of object investigation time has occurred (e.g. Clark, Zola, & Squire, 2000). Following the retention delay, which can vary between a few minutes to days, the rodent is returned to the open field for the test phase. During this phase, the rodent is presented with a third copy of the object present during familiarization (i.e. familiar object) and a novel one. Again, the amount of time the rodent explores each object is recorded. *Figure 1.4* presents a schematic diagram depicting the procedure for the NOP test. Rodents will typically spend more time with the novel relative to the familiar object following retention delays lasting several minutes to hours (Clark et al., 2000; Ennaceur & Delacour, 1988; Ennaceur & Aggleton, 1994). This novelty preference requires recognition of the familiar object and for this reason, the NOP test performance is believed to reflect ORM ability.

Novelty preference is typically calculated as a ratio of time spent investigating the novel object over the time spent investigating both objects. When calculated in this way, a preference for the novel object is reflected in a ratio larger than 0.5 (i.e. equal time with both objects). Within-group comparisons are made by comparing the group mean to 0.5 and when they are significantly

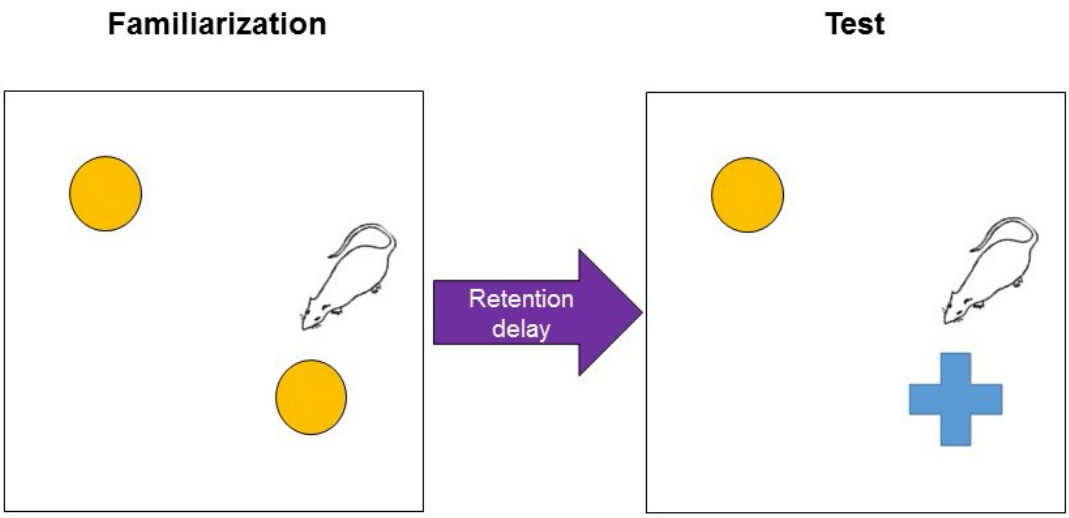

Figure 1.4. Schematic representation of Novel-Object Preference developed by Ennaceur and Delacour (1988).

above chance-level, are said to reflect a preference for the novel object. Otherwise, ratios reflect no preference, or in some cases, a preference for the sample object (when the ratio is significantly below chance). Other researchers calculate a difference score and rather than comparing to 0.5, scores are compared to 0. Ratio or difference scores are used as the dependent measure rather than time spent with each object as it removes individual variability in investigation time, which would increase error variance. In either case, within-group rather than between-group comparisons are made as they allow researchers to determine whether or not each group demonstrates novelty preference.

The NOP test has become one of the most widely used rodent behavioural tests (Lyon, Saksida, & Bussey, 2012), including studies that investigate the role of the HPC and PRh in ORM. The first study to investigate disruptions in HPC function on NOP test performance (Ennaceur & Aggleton, 1994) found no evidence of an impairment, as both fornix-lesioned and control rats demonstrated novelty preference following retention delays of 1 min and 15 min, but not 4 hr. Subsequent studies also found that rats with fornix lesions demonstrated novelty preference (for a review, see Mumby, 2001). The majority of later studies that examined the effect of direct HPC lesions on NOP test performance also found intact novelty preference following delays as long as 24 hr (Forwood, Winters, & Bussey, 2005; Gaskin, Tremblay, & Mumby, 2003; Gaskin et al., 2010; Good, Barnes, Staal, McGregor, & Honey, 2007; Langston & Wood, 2010; Mumby, Glenn, Nesbitt, & Kyriazis, 2002; Mumby, Tremblay,

Lecluse, & Lehmann, 2005; Winters, Forwood, Cowell, Saksida, & Bussey, 2004). Although some studies do report disrupted NOP test performance following HPC lesions (Clark et al., 2000, Clark, West, Zola, & Squire, 2001), the evidence does not convincingly support a role of the HPC in novelty preference or ORM (Winters et al., 2008).

The NOP test has also been used to examine the role of the PRh in novelty preference. In contrast to the HPC, several studies have found that PRh function is important for novelty preference (Bussey, Muir, & Aggleton, 1999; Ennaceur, Neave, & Aggleton, 1996; Mumby et al., 2002, Mumby, Piterkin, Lecluse, & Lehmann, 2007). Mumby and colleagues (2002) examined the effects of PRh lesions on retrograde and anterograde memory. Rats were exposed to a sample object 5, 3, or 1 week before surgery. When tested after surgery, the control animals exposed to the sample object 3 or 1 week prior to surgery demonstrated novelty preference on the post-surgery test, whereas none of the PRh-lesioned groups demonstrated a preference. These same animals were then exposed to a different sample object and when tested 15 min later, the PRh-lesioned rats again demonstrated no preference whereas novelty preference was observed in the control group. Unlike the HPC, the PRh appears to play a role in novelty preference.

1.4.3 Comparison between DNMS task and NOP test. Each behavioral test has certain advantages and disadvantages associated with their use. This is true for both the DNMS task and NOP test. When using trial-unique stimuli, the DNMS task requires several hundred training trials before a performance

criterion is reached, which is taxing on the experimenter. Another disadvantage involves interpreting impaired performance. In order for the rodent to perform reliably on this task, it must be able to recognize the familiar object. However, it must also learn the association between novelty and reward and so a failure to reliably choose the novel over familiar object may reflect an impairment in either ability. This is typically overcome by having test trials with several different retention delays, with the shortest delay being identical to the one used during the training phase. The remaining test trials use increasingly longer retention delays. If a rat demonstrates impaired performance following all retention delays, then this impairment may be due to the inability to recognize the familiar object or the inability to apply the rule learnt during training. However, if a rat were to demonstrate intact performance following shorter delays, but impaired performance following longer delays, then it is unlikely that it is unable to apply the previously acquired rule. Rather, it is more likely that it is no longer able to recognize the sample object (following long retention delays). Another disadvantage of the DNMS task is that it requires the rodent to be in a certain motivational state that makes the food pellet rewarding. Again, if an experimental manipulation disrupted the motivational state during testing, it would likely impact performance regardless of retention delay.

The main benefits of the NOP test is that the observed behavior (i.e. object investigation) requires no training, nor does it require the rodent to learn or apply an associative rule. The main disadvantage concerns understanding why a treatment group fails to demonstrate novelty preference. Since novelty

preference requires intact ORM ability, most researchers interpret a failure to demonstrate novelty preference as a memory impairment. However, a lack of preference for the novel object may occur despite intact ORM. For example, Gervais, Jacob, Brake and Mumby (2013) conducted a study comparing ovariectomized rats given intra-PRh/EC infusion of 17-β estradiol (E2) or vehicle on the NOP test and DNMS task. While the vehicle-treated rats failed to demonstrate novelty preference, their accuracy on the DNMS task was better than the E2-treated condition. In other words, despite intact ORM, vehicle-treated rats failed to demonstrate novelty preference. Therefore, any treatment effect on novelty preference may reflect an influence on ORM ability, or on non-cognitive factors that contribute to the behavioural expression of this preference. Incorporating NOP test trials with short and long retention delays may help disambiguate a treatment effect on novelty preference in the same way that it does for the DNMS task. For example, a failure to demonstrate novelty preference following a long, but not short retention delay is consistent with a memory impairment.

Another issue limiting use of the NOP test concerns the magnitude of the novelty preference. As stated above, within-group comparisons on novelty preference are typically made and interpreted as reflecting either a preference for the novel object, or no preference. However, some investigators compare the magnitude of the novelty preference across groups. For example, Clark and colleagues (2000) report lower preference ratios in rats with HPC lesions compared to controls following retention delays of 10 min to 24 hr. The authors

interpreted the lower preference ratios as reflecting an ORM impairment. However, recent evidence suggests that preferences higher in magnitude may not reflect better ORM (Gaskin et al., 2010). Therefore, caution is needed when explaining a treatment effect on both the failure to demonstrate novelty preference, and on the magnitude of the preference (when between-group comparisons are made).

Although there are disadvantages to using the nonrecurring-items DNMS task, one major advantage is that accuracy scores likely reflect the strength of the ORM ability. Therefore, performance on the DNMS task can inform us with greater certainty whether a particular treatment influences the strength of the ORM.

The NOP test can provide useful information regarding ORM ability when used in conjunction with the DNMS task. For example, an experimental treatment that results in impaired DNMS task performance and no preference on the NOP test is suggestive of an effect on ORM ability. However, in the case when a study calls for the use of retention delays longer than what is possible with the DNMS task (which typically involves retention delays lasting seconds to minutes), the NOP test is the only option. In this situation, multiple retention delays can be used to determine whether a delay-dependent effect exists, as this is consistent with an effect on memory ability.

1.4.4 Conclusions regarding use of rodents to model MTL amnesia.

Developing ORM tests for use with rodents is an important contribution to understanding the role of MTL structures in this ability. Starting in the mid-1980s,

tests comparable to those used in macaques were introduced for rodents. Consistent with the macaque literature, studies in rats suggest the HPC has little or no role in ORM. Rather, as suggested by research on macaques, the PRh is important for this ability.

1.5 Mechanisms of PRh-mediated ORM

1.5.1 Neuronal responses to familiarity. *In vivo* recordings of neurons in the PRh and the temporal association cortex (TeA) in macaques demonstrate a reduction in the firing rate following exposure to familiar relative to novel visual stimuli (Brown & Aggleton, 2001). This observation offers a potential substrate through which the PRh may process the familiarity of objects previously encountered. Such responses are also seen in rats (Zhu, Brown, & Aggleton, 1995**).** Decreased neuronal activation following familiar relative to novel stimuli is also observed. For example, Zhu, Brown, McCabe and Aggleton (1995) examined Fos expression in rats following exploration of either a familiar or novel object. C-fos is an immediate early gene (IEG) and its protein, Fos, is increasingly expressed in neurons during periods of higher firing. Thus, c-fos, or Fos, are considered markers of neuronal activity. Zhu, Brown, McCabe and colleagues (1995) reported less Fos-immunoreactivity (IR) in both the PRh and TeA following presentation of a highly familiar object relative to a novel one, suggesting that reduced neuronal activity in the PRh and TeA is involved in ORM. A similar pattern of results is reported in studies using inter-hemispheric comparisons involving the presentation of different stimuli in each eye and observing Fos-IR in brain regions on the contralateral side (Zhu et al., 1996; Wan

et al., 1999). These studies describe reduced activity in the PRh and TeA following familiar- relative to novel-stimulus presentation, suggesting that reduced PRh activity may reflect how familiar objects are recognized (i.e. familiarity discrimination).

1.5.2 Synaptic plasticity in the PRh following exposure to familiar and novel stimuli. As described in Section 1.1.2, PRh neurons are capable of both LTP and LTD. However, Brown and Bashir (2002) argue that LTD is a better model for PRh-mediated ORM. Massey and colleagues (2008) examined the relationship between depotentiation and LTD, and novel-stimulus related neuronal responding in the PRh. Depotentiation is believed to involve similar processes as LTD, however, it is induced following LTP. Rats were exposed to visual stimuli using the inter-hemispheric technique described in Section 1.5.1. Following stimulus presentation, brain slices from both hemispheres were obtained and LTD and depotentiation were induced. Depotentiation and LTD were both prevented in the slices on the contralateral side exposed to familiar stimuli. In contrast, both depotentiation and LTD were induced in slices on the contralateral exposed to the novel stimuli. LTP was induced in all slices. The results from this experiment are consistent with the idea that depotentiation and LTD, but not LTP, model synaptic changes that promote familiarity detection, and perhaps ORM.

1.5.3 Potential molecular mechanisms of PRh-mediated ORM. The majority of research investigating molecular mechanisms of ORM focus on the HPC (Winters et al., 2008). This is counterintuitive considering the lack of support for

this region in ORM (see sections 1.2-1.4 above). Since this thesis involves an examination of modulators in the PRh, and potential neuronal correlates they influence to promote ORM, only studies reporting evidence for PRh-mediated mechanisms of this ability will be reviewed.

Warburton and colleagues (2005) examined the role of the phosphorylated (i.e. active) form of cAMP response element binding protein (pCREB) in novelty preference and neuronal responding to familiar and novel objects. CREB is a transcription factor that is activated during signaling cascades associated with cellular memory consolidation (Kandel, 2001). Phosphorylated CREB binds to the cAMP response element (CRE) located on the promoter region of target genes. This cascade results in the synthesis of proteins, some of which alter the morphology and function of the neuron in ways thought to be important for memory formation. In the study by Warburton and colleagues (2005), novelty preference was assessed using NOP test trials with 2 retention delays (15 min and 24 hr). Inhibiting pCREB prevented the expression of novelty preference following a 24-hr but not 15-min retention delay. In addition, this inhibition prevented reduced neuronal activation in the PRh following familiar stimulus presentation. The results suggest that pCREB in the PRh is associated with novelty preference and neuronal activation related to novel-stimulus presentation.

Activation of mitogen activated protein kinases (MAPK) is a step upstream in the signaling cascade that contributes to the phosphorylation of CREB (Kandel, 2001). A recent study (Silingardi et al., 2011) investigated

whether phosphorylated MAPK in the PRh is necessary for novelty preference. Immediately following the familiarization phase, bilateral intra-PRh infusions of either a MAPK inhibitor (UO126) or vehicle were given. Twelve hours later, the mice returned to the open field for the test phase. Novelty preference was observed in the vehicle but not treatment condition, suggesting a role of intra-PRh MAPK in ORM.

Brain-derived neurotropic factor (BDNF) is a protein implicated in cellular consolidation (Callaghan & Kelly, 2012) and synaptic plasticity (Seoane, Tinsley, & Brown, 2011). Specifically, BDNF is known to initiate the MAPK pathway discussed above, and also the PI3K/Akt pathway. In one experiment (Seoane et al., 2011), the role of PRh-BDNF in novelty preference was investigated. Two treatments were infused locally in the PRh, one was a BDNF antisense oligodeoxynucleotide (ODN) used to interfere with BDNF expression. The second infusion type was a sense ODN and served as a control condition. Infusions occurred either immediately before the familiarization phase of the NOP test, immediately after, 6 h after, or 1 hr before the test phase. The retention delays were either 20 min, 24 hr, or 30 hr. The antisense ODN did not influence novelty preference following a 20-min retention delay, but did following a 24-hr delay. Specifically, the antisense resulted in no preference on the 24-hr retention test when infused either immediately before or after familiarization. The antisense had no effect when infused 6 hr following familiarization, or 1 hr before the test phase. The control condition demonstrated novelty preference during all trials. These findings support the idea that BDNF modulates ORM.

In summary, there are a few studies demonstrating a role of PRh-BDNF and the MAPK signaling cascade in novelty preference. Yet, the studies available used exclusively the NOP test. It remains to be seen whether BDNF and MAPK signaling also influence DNMS task performance.

1.6 Modulators of ORM

As mentioned in Section 1.1.2, the PRh is capable of glutamatergic, cholinergic, dopaminergic, adrenergic and serotonergic neurotransmission (Kealy & Commins, 2011). Glu is known to play a central role in synaptic plasticity (Winters et al., 2008). In particular, the NMDAR plays a central role in LTP (Kandel, 2001). In the PRh, LTD requires co-activation of both NMDAR and Glu metabotropic receptors (Brown & Bashir, 2002). In addition, results from studies using the NOP test demonstrate that NMDAR and AMPAR modulate novelty preference (Winters & Bussey, 2005b). Taken together, these studies provide evidence for a role of Glu in PRh-mediated memory processes.

Other than Glu, ACh is also in involved in both PRh synaptic plasticity and ORM.

1.6.1. Acetylcholine (ACh). ACh is synthesized in motor neurons of the spinal cord, brain stem, and basal forebrain. Synthesis occurs in the presence of choline acetyltransferase (ChAT). This enzyme is present only in neurons that release ACh and so is used as a marker for cholinergic neurons. It is produced in the soma, then transported to the terminal, where it transfers the acetyl group from acetyl Coenzyme A to choline to make ACh. ACh is then stored in vesicles until it is released in the synapse. Acetylcholinesterase is also produced by

cholinergic neurons and is released into the synaptic cleft where it catabolizes ACh into choline and acetic acid (Bear et al., 2007).

In the brain, there are two sources of cholinergic neurons, one originating from the pons and midbrain (i.e. pontomesencephalotegmental complex). These neurons provide cholinergic input to the thalamus and also innervate the telencephalon. The second major input originates in the basal forebrain complex. This complex, which includes the medial septal nuclei, innervates the MTL region, and the basal nucleus of Meynert, which projects to the neocortex (Bear et al., 2007).

Damage to cholinergic afferents from the medial septum (of the basal forebrain) to the PRh prevents novelty preference. Winters and Bussey (2005a) damaged these projections via direct infusion of the immunotoxin 192 IgG-saporin into the PRh of rats. This immunotoxin produces selective damage to basal forebrain cholinergic projections by acting on a receptor for the nerve growth factor 192 IgG (p75 NGFr). This receptor is highly expressed in ACh neurons in the basal forebrain compared to other cholinergic neurons and non-cholinergic neurons (Ricceri, 2003). Histological analysis using ChAT expression confirmed that the selective lesions resulted in loss of cholinergic neurons in the basal forebrain. Behavioral analysis included the NOP test and a spatial alternation test, for which performance is not believed to require the PRh. Results suggest a functional dissociation as the lesions disrupted performance on the NOP test, but not the spatial alternation test. A second study reported performance deficits in macaques on the DNMS task following selective damage

to the cholinergic afferents to the PRh and EC (Turchi, Saunders, & Mishkin, 2005). Further evidence for a role of PRh-ACh in ORM comes from studies demonstrating higher levels of ACh release in the PRh while macaques perform the DNMS task compared to levels obtained prior to starting the task. (Tang & Aigner, 1996). Taken together, these studies suggest that ACh release in the PRh via the basal forebrain projections modulates ORM.

There are two cholinergic receptor types, nicotinic and muscarinic, and both are present in the PRh (Kealy & Commins, 2011). Several studies using pharmacological manipulations have found support for a role of muscarinic receptors (mAChR) in PRh-mediated novelty preference. Systemic administration of scopolamine or atropine, both of which are mAChR antagonists, prevents novelty preference in rats (Bartolini, Casamenti, & Pepeu, 1996; Ennaceur & Meliani, 1992; Huston & Aggleton, 1987; Pitsikas et al., 2001; Vannucchi, Scali, Kopf, Pepeu, & Casamenti, 1997), visual recognition memory in humans, and ORM in macaques (Robbins et al., 1997; Aigner & Mishkin, 1986; Aigner, Walker, & Mishkin, 1991; Penetar & McDonough, 1983). Intra-PRh infusion of scopolamine also prevents novelty preference, although only when infused prior to, and not after the familiarization phase (Warburton et al., 2003; Winters, Saksida, & Bussey, 2006). MAChR is also involved in the observed decrease in neuronal responding to familiar stimuli, as systemic administration of scopolamine prevents reduced Fos-IR in the PRh following familiar- relative to novel-stimulus presentation. Scopolamine also prevents LTD induction in PRh neurons (Warburton et al., 2003). The most convincing evidence for a role of

intra-PRh mAChR in ORM comes from a study demonstrating impaired DNMS task performance in macaques following local infusions of scopolamine relative to vehicle (Tang, Mishkin, & Aigner, 1997). In summary, there is evidence suggesting that mAChR in the PRh is important for novelty preference and that this may extend to ORM. However, there are not many studies investigating a role for this receptor type in consolidation of object representations. Furthermore, memory function in females has been shown to be affected by ovarian hormones and their role in the PRh and ORM remains virtually unexplored.

1.6.2 Estrogens

Estrogen (E) is a class of gonadal steroid hormones, known to play several important roles in sexual development, reproduction, and behaviour (Bear et al., 2007). There are multiple types of E, including 17β-estradiol, estrone, and estriol, all of which easily enter the bloodstream where they travel to target tissues (Nelson, 2005), including the brain where they easily cross the blood-brain barrier. Among the different forms of E, 17β-estradiol (E2) is the most common and potent during the reproductive years in females.

E2, like all steroid hormones, is synthesized from cholesterol, which is converted to pregnenolone. In the gonads, pregnenolone is converted to a class of steroid hormones called androgens (e.g. testosterone), which is then converted to E2 in the ovaries. E2 is also synthesized in the brain in both males and females (Hojo et al., 2008). In females, E2 and other ovarian hormones fluctuate cyclically (Nelson, 2005). In humans and non-human primates, this cycle is referred to as the menstrual cycle as it consists of hormonal fluctuations

accompanied by menstruation (i.e. discarding the uterine endometrium). The cycle consists of three phases. During the first phase, menses, menstruation occurs and coincides with maturation of an ovum. This is also when E2 and progesterone levels are at their lowest. The follicular phase follows the end of menstruation and consists of a rise in E2 as well as development of the follicles. This phase ends with ovulation, or release of an egg, when E2 has peaked. The final phase, luteal, consists of development of the corpus lutea, which forms from follicles and results in a large increase in progesterone levels. Progesterone then drops at the end of this phase right before menstruation. E2 levels remain low throughout this phase. In rodents and other species that do not experience menses, this cycle is referred to as the estrous cycle. The estrous cycle of laboratory rats lasts between 4-5 days and also consists of three phases. The first phase, estrous, is when E2 levels are lowest, progesterone decreases, and when follicles begin to develop. This phase is followed by diestrous. Since this phase persists for approximately 48 hours, it is further subdivided into diestrous 1 (i.e. metestrous) and diestrous 2. E2 levels during this phase begin to increase, although progesterone remains low. By the end of this phase, follicles are enlarged and referred to as Graafian cells. The corpora lutea is also now fully developed. During the final phase, proestrous, E2 levels peak, followed by progesterone 12 hours later, which coincides with the onset of proceptive behaviours, or behaviours involved in initiating copulation (Beach, 1976).

1.6.2.2 E modulates ORM. In addition to sexual behaviour, E2 has been shown to both enhance and impair learning and memory abilities across species (Korol,

2004; Sherwin, 2012; Rapp, Morrison, & Roberts, 2003), and the direction of the effect appears to depend on a number of factors, including the ability being assessed, dose, interactions with other hormones, duration of treatment, mode of delivery, sex, stress, and motivation (Korol, 2004; Zurkovsky, Brown, Boyd, Fell, & Korol, 2007).

The role of E2 in ORM has been studied in humans, non-human primates, and rodents. Studies using both humans and non-human primates have found that surgical or pharmacologically-induced ovariectomy (OVX) impairs performance on the DNMS or delayed matching-to-sample (DMS) tasks relative to an E replacement condition (Craig et al., 2010; Lacreuse, Herndon, & Moss, 2000; Voytko, Higgs, & Murray, 2008), although some have reported no effect (Lacreuse & Herndon, 2003). Studies in both rats and mice demonstrate that E2 replacement enhances novelty preference relative to no E2 replacement (Fernandez & Frick, 2004; Gresack & Frick, 2006; Inagaki, Gautreaux, & Luine, 2010; Luine, Jacome, & Maclusky, 2003). Some data suggest that E2 acts during consolidation to enhance novelty preference. Whether or not E2 replacement also enhances DNMS task performance in rodents remains to be seen.

It is currently unknown whether E2 acts within the PRh to modulate ORM. However, there is some evidence that E2 has actions within this brain region.

1.6.2.3 Estrogen receptors (ERs) in the PRh. There are currently 3 known estrogen receptors (ERs) in the brain, ER alpha (ERα) ER beta (ERβ) and G protein-coupled ER 1 (GPER1). GPER1 is a membrane-bound receptor, while

ERα and ERβ are located both along the membrane and in the nucleus (Spencer et al., 2008; Frick, Fernandez, & Harburger, 2010).

E2 has both slow (hours to days) and rapid (< 1 hr) actions in the brain. The slow actions include changes in gene expression, including promoting the synthesis of glutamic acid decarboxylase (GAD; Spencer et al., 2008), an enzyme involved in converting Glu to GABA. Rapid effects of E2 include activation of G proteins, growth factor receptors, second messenger cascades (i.e. cAMP) and intracellular kinases, including PKA and MAPK (Korol & Gold, 2007; Spencer et al., 2008). Since E2 replacement enhances novelty preference when administered immediately before and immediately after, but not 2 hr following the familiarization phase (Luine et al., 2003), it is believed that E2 acts rapidly to promote novelty preference.

ERβ, but not ERα is implicated in novelty preference (Jacome et al., 2010; Walf, Koonce, & Frye, 2008; Walf, Koonce, Manley, & Frye, 2009). For example, systemic administration of diarylpropionitrile (DPN), a selective ERβ agonist, to OVX rats resulted in novelty preference whereas administration of propyl pyrazole triol (PPT), a selective ERα agonist, or vehicle did not. The novelty preference observed in the DPN-treatment condition occurred when the drug was administered before and immediately following familiarization (Jacome et al., 2010), which supports the idea that ERβ is involved in consolidation. However, one study reports that a selective agonist to ERα, but not ERβ, enhances novelty preference in OVX mice (Phan, Lancaster, Armstrong, Maclusky, & Choleris, 2011), and another that reports no effect of either ligand on DNMS task

performance in macaques (Lacreuse, Wilson, & Herndon, 2009). Currently, there are no published studies addressing the role of GPER1 in ORM. Given the current lack of conclusive data, it is unclear which ERs are involved in ORM.

The distribution of the three ERs in the brain has been characterized in several studies, and all 3 are expressed in the PRh (Blurton-Jones & Tuszynski, 2002; Brailoiu et al., 2007; Hazell et al., 2009; Shughrue, Lane, & Merchenthaler, 1997; Shughrue & Merchenthaler, 2001). Since ERβ is implicated in novelty preference, and is expressed in the PRh, it is possible that E2 binds to this receptor in the PRh to influence novelty preference, and perhaps ORM. However, it is also possible the other ERs influence PRh-mediated ORM.

Currently, there is little research that addresses the modulatory effect of E2 on neuronal function in the PRh. However, several studies support such a role on neuronal function and plasticity within another brain region implicated in learning and memory, namely the HPC.

1.6.2.4 E2 influences neuronal activation and synaptic plasticity

Fonseca and colleagues (2013) examined neuronal activation following novel-object or open field exploration. OVX mice received chronic E2 replacement (0.18 mg/4 µl in corn oil, via silastic implant) or no replacement prior to novel-object exploration in an open field arena, or a no object control condition. Following exploration, Fos-IR was examined. Regardless of object exploration, E2 replacement resulted in more Fos-IR cells in the PRh, HPC, and amygdala. Within the E2 replacement condition, exploration of objects was associated with more neuronal activation than exposure to the open field alone.

While this study provides preliminary evidence that E2 influences PRh activity, there are some important limitations to the study by Fonseca and colleagues (2013) to consider. E2 is known to influence ambulatory activity levels of rodents (Archer, 1975), and so any increase in exploration resulting from elevated levels of E2 can explain the augmented Fos expression in the E2 replacement group. Ambulation was not compared across the two conditions, so this possibility cannot be ruled out. Fonseca and colleagues (2013) also conducted within-group comparisons that did not include an appropriate control group. While it is certainly useful to have a control group in which no objects are present, it is equally important to include another condition in which the objects being presented are familiar. Previous studies investigating PRh activation following stimulus presentation observe more Fos-IR cells following novel- compared to familiar-stimulus presentation (Albasser, Poirier, & Aggleton, 2010; Wan, Aggleton, & Brown, 1999; Warburton et al., 2003; Zhu, Brown, McCabe, & Aggleton, 1995). Thus, the increased Fos-IR observed by Fonseca and colleagues (2013) within the E2 replacement condition may have been due to the presence of additional stimuli (i.e. objects) rather than encoding of object representations. Although chronic E2 replacement influences neuronal activation in the PRh, it is difficult to reconcile this effect with a role in PRh-mediated ORM.

Evidence from studies examining mechanisms in the HPC provides support for the idea that E2 modulates excitatory synaptic transmission and synaptogenesis. For example, the density of dendritic spines, which provide the majority of excitatory synapses in this region, fluctuate across the estrous phase,

with the greatest density during proestrous, and lowest during estrous (Woolley & McEwen, 1992). OVX results in decreased spine density (Gould, Woolley, Frankfurt, & McEwen, 1990), while high E replacement (10 μg, EB in 0.1 ml oil, s.c.) prevents the decrease following OVX (Woolley & McEwen, 1993). These results suggest that higher levels of E2 are associated with a greater density of dendritic spines. Since the majority of excitatory synapses form on these spines (Yosihara, De Roo, & Muller, 2009), higher E2 is associated with greater density of excitatory synapses. Higher levels of E2 are also associated with an augmentation of LTP induction, both in gonadally-intact rats (Good, Day, & Muir, 1999), and following E2 replacement in OVX rats (Córdoba, Montoya, & Carrer 1997). An increase in the expression of synaptic proteins is also observed in the HPC following higher levels of E2 (Brake et al., 2001; Frick, Fernandez, & Bulinski, 2002). These studies provide evidence for an association between higher E2 levels and increased excitatory synaptic transmission in the HPC.

There is also evidence that E2 influences ACh neurotransmission in the HPC by acting directly on projections from the basal forebrain (Hammond, Nelson, & Gibbs, 2011; Spencer et al., 2008). These fibres are thought to be necessary for the observed disinhibition of HPC neurons that occurs following E2 administration (Rudick, Gibbs, & Woolley, 2003). Administration of an acetylcholinetransferase inhibitor mimics the facilitation of E2 on NMDAR binding (Daniel & Dohanich, 2001). Therefore, E2 appears to influence both Glu and ACh neurotransmission. Given that both neurotransmitters are implicated in PRh-

mediated ORM, and given that E2 might also influence this ability, it may do so by mediating Glu and ACh neurotransmission in the PRh.

1.7 Summary and rationale

Macaques and rodents demonstrate ORM (Mishkin, 1978; Mumby et al., 1990) which resembles human visual recognition memory, and so these species have been used to investigate the contribution of different MTL structures to this ability. While previous studies confirm a role of the PRh in ORM (Winters et al., 2008), less support exists for a role of the HPC (Mumby, 2001). Despite the lack of support, several investigations into potential mechanisms of ORM have concentrated on the HPC, with less research focusing on the PRh (Winters et al., 2008). For this reason, little is known about neuronal correlates of ORM.

There is evidence supporting that modulators of ORM act within the PRh. For example. ACh has been shown to modulate ORM by acting within the PRh (Winters et al., 2008). All types of ACh receptors are expressed in this region (Kealy & Commins, 2011), but mAChR is the receptor type that has received the most attention for its role in ORM. While the majority of research suggests mAChR plays a role during familiarization (Warburton et al., 2003; Winters et al., 2006), less is known about its role in consolidation. The majority of studies examining the involvement of this receptor type in ORM have used the NOP test using short retention delays (1-60 min; Bartolini et al., 1996; Ennaceur & Meliani, 1992; Pitsikas et al., 2001; Vannucchi et al., 1997), with few studies using delays longer than 60 min (Tinsley et a., 2011; Winters et al., 2006), and only one study reporting a delay-dependent effect (Tinsley et al., 2011). Therefore, an important

next step is to determine whether intra-PRh mAChR influences novelty preference by acting during encoding of object representations (during familiarization) following retention delays longer than 60 min, and whether the effect extends to consolidation. Given the limitations of the NOP test discussed in Section 1.4.3, two different retention delays are required. The short delay should be longer than 60 min, but brief enough to reliably result in novelty preference, while the other delay should be long enough to result in novelty preference just slightly above chance. This was addressed in the study described in Chapter 2 of the present thesis. In that study, intra-PRh infusions of either atropine sulfate (100 μM) or vehicle were given before or after the familiarization phase of the NOP test using a 4- and 24-hr retention delay.

E2 is a hormone thought to modulate processes involved in ORM. Systemic administration of E2 enhances DNMS task performance in macaques (Rapp et al., 2003) and novelty preference in rodents (Luine et al., 2003). There is some evidence suggesting E2 affects novelty preference by influencing the consolidation of object representations (Luine et al., 2003). However, it remains to be seen whether it acts within the PRh to alter this ability. The majority of previous research compares ovariectomized (OVX) females with E2 replacement to those without replacement. However, it is unknown whether the pattern of results changes when the comparison group experiences physiologically-relevant, low levels of E2. Chapter 3 addresses whether acute high E2 replacement enhances ORM by comparing two E replacement conditions on both the NOP test and DNMS task, 1) chronic low E2 and acute high E replacement,

and 2) chronic low E2 replacement. Chapter 3 also addresses whether DNMS task and NOP test performance is influenced by intra-PRh/EC infusion of E2 in OVX rats with chronic low E2 replacement.

All three ERs are expressed in the PRh (Blurton-Jones & Tuszynski, 2002; Brailoiu et al., 2007; Hazell et al., 2009; Shughrue et al.,1997; Shughrue & Merchenthaler, 2001), and there is some support that ERβ influences novelty preference (Jacome et al., 2010; Walf et al., 2008, 2009). However, it remains to be seen whether ERβ agonists influence ORM. The study described in Chapter 4 addresses whether intra-PRh infusion of DPN (2 μg/μl), a selective ERβ agonist influences NOP test and DNMS task performance. E2 and vehicle were also administered. Similar to the experiment described in Chapter 2, two retention delays were used on the NOP test to determine whether any effects of either E2 or DPN are delay-dependent. Taken together, Chapters 2-4 address the primary aim of the present thesis, which is to examine the contribution of intra-PRh ACh and E2 on ORM.

In addition to ORM, ACh and E2 also influence neuronal correlates of learning and memory (Warburton et al., 2003; Woolley, 1998). Reduced neuronal firing in the PRh following presentation of familiar stimuli is thought to be the neuronal basis of familiarity discrimination (Brown & Aggleton, 2001). There is some evidence that mAChR is involved in the initial responses of PRh to novel visual stimuli (Warburton et al., 2003). However, it remains to be seen whether a similar role is observed following exploration of novel objects. This is particularly important, as rats acquire information about objects mainly from olfactory rather

than visual cues. Given evidence that a35 and a36 are functionally distinct, it remains to be seen whether any effect of mAChR on neuronal activation differs in these two sub-regions. Finally, the posterior portion of the PRh rather than more anterior regions, is implicated in ORM (Albasser et al., 2010). These issues are addressed in the study described in Chapter 2. Rats received intra-PRh infusion of atropine sulfate (100 μM) or vehicle prior to familiar- or novel-object exploration. Their brains were then processed for immunohistochemical analysis of Fos expression, a marker of neuronal activation. The number of Fos-IR cells in a35 and a36 in the anterior, middle, and posterior PRh was compared across the two groups. To test whether neuronal activation (as measures by Fos-IR) following novel-object exploration reflects a neuronal correlate of ORM, correlations were conducted between the number of Fos-IR cells in each regions and performance on the NOP test.

Changes in Glu synaptic transmission and synaptogenesis in the HPC are also considered neuronal correlates of learning and memory (Woolley, 1998). These changes include larger responses to excitatory input (Wong & Moss, 1992), lowered threshold to induced LTP (Córdoba et al., 1997), increased expression of synaptic proteins (Brake et al., 2001), and greater density of dendritic spines (Woolley & McEwen, 1992), which form the majority of excitatory synapses in the CNS (Yosihara et al., 2009). In the PRh, Glu synaptic transmission is implicated in both novelty preference (Winters et al., 2005b) and LTD (Banks, Bashir, & Brown, 2012), a model of long-term memory formation implicated in ORM. While higher E2 levels are associated with greater Glu

synaptic transmission and synaptogenesis in the HPC (Woolley, 1998), it remains to be seen whether they are also associated in the PRh. The study described in Chapter 5 addresses this by examining whether endogenous elevations of E2 and high E2 replacement (10 μg/kg, s.c.) are associated with greater density of dendritic spines in a35 and a36. This final study of the thesis helps determine whether actions of E2 within the PRh influence morphological changes within this region that may promote ORM. Taken together, Chapters 2 and 5 address the second aim of the present thesis, which is to define how ACh and E2 influence neuronal correlates of ORM.

1.8 Hypotheses

The aims of this thesis are to conduct an evaluation of both ACh and E2 in ORM, neuronal function and Glu synaptic density in the PRh. Chapter 2 addresses the role of one type of cholinergic receptors, mAChR, in ORM and novel-object exploration-related PRh activation. Intra-PRh administration of scopolamine, a mAChR antagonist, prevents novelty preference when infused prior to familiarization, and prevents reduced firing of PRh neurons when infused prior to repeated exposure to familiar visual stimuli. Therefore, it was hypothesized that mAChR antagonism (via intra-PRh infusion of atropine sulfate; 100 μM) prevents novelty preference when infused prior to, but not following familiarization. Finally, it was expected that exploration of novel objects would result in more Fos-IR cells in the posterior PRh, and that mAChR antagonism (via intra-PRh infusions of atropine) would prevent this increase. The number of Fos-IR cells were predicted to correlate positively with the magnitude of novelty

preference. While novelty preference was prevented by intra-PRh infusion of atropine, the effect was not delay-dependent. More Fos-IR neurons in the posterior a36 was observed following novel- relative to familiar-object exploration, which was influenced by mAChR antagonism. However, the number of Fos-IR cells in this portion of the PRh was uncorrelated with novelty preference.

Chapter 3 addresses the role of E2 in ORM, and whether it acts within the PRh to influence this ability. Higher levels of E2, and intra-rhinal cortical E2 was hypothesized to improve ORM, reflected in enhanced novelty preference following a 72-hr retention delay, and better accuracy on the nonrecurring-items DNMS task following longer, but not shorter, retention delays. Results confirm enhanced novelty preference following acute high E replacement, and intra-rhinal cortical infusion of E2 occurring either immediately before or after familiarization, but not 2 hr later. In contrast, lower accuracy on the DNMS task was observed following intra-rhinal cortical infusion of E2 relative to vehicle infusion at a 3-min retention delay. Chapter 4 addresses whether E2 binding to ERβ in the PRh influences performance on the NOP test and DNMS task. Two retention delays were used on the NOP test to determine whether a delay-dependent effect would occur. It was predicted that intra-PRh infusion of E2 and DPN (a selective ERβ agonist) would result in enhanced novelty preference following the 72-hr, but not 4-hr, retention delay. Similar to the results in Chapter 3, novelty preference was enhanced by intra-PRh infusion of DPN and E2. The enhanced novelty preference following DPN infusions was unaffected by retention delay. Delay-

dependent impairments were observed on the DNMS task following intra-PRh infusion of E2, but not DPN.

The final experiment, which is presented in Chapter 5, addresses whether synaptic (or dendritic spine) density in the PRh changes following elevations in E2 levels. This was addressed in gonadally-intact female rats and in OVX rats with and without E2 replacement. While no differences were observed between rats in proestrous and estrous, decreases in the density of mature spines in a35 were observed following high E2 replacement (10 μg/kg, s.c.) relative to no replacement.

CHAPTER 2

Muscarinic receptors in the perirhinal cortex influence novelty preference and neuronal activation

Abstract

Reduced neuronal activation in the perirhinal cortex (PRh) following presentation of familiar relative to novel stimuli is a proposed mechanism for familiarity discrimination (Brown & Aggleton, 2001), or object-recognition memory (ORM). Muscarinic receptor (mAChR) antagonism during repeated presentation of familiar visual stimuli prevents this reduced activation in the PRh. Therefore, it is proposed that these receptors modulate ORM via this neuronal mechanism in the PRh. One goal of the present study was to determine whether a mAChR antagonist prevents novelty preference in a delay-dependent manner, and reduces neuronal activation following novel-object exploration. While the majority of research supports the involvement of mAChR in the familiarization to a sample object, less is known about their role in consolidation. Thus, a second goal was to examine whether a mAChR antagonist prevents novelty preference when administered before and after familiarization. Intra-PRh infusion of atropine sulfate (100 μM) prevented novelty preference regardless of retention delay and timing of drug administration. Consistent with the neuronal mechanism of familiarity discrimination, novel-object exploration resulted in more Fos-immunoreactivity in the posterior a36 subregion of the PRh relative to exploration of familiar objects. This increase in neuronal activation was attenuated by an intra-PRh infusion of atropine. The magnitude of novelty preference was not correlated with Fos expression. These results suggest that ACh binds to mAChR in the PRh to promote both novelty preference and novelty-related neuronal activation. The present study provides some support for the neuronal basis of familiarity discrimination proposed by Brown & Aggleton (2001).

2.1 Introduction

Acetylcholine in the PRh is implicated in object-recognition memory (ORM; Turchi, Saunders, & Mishkin, 2005; Winters & Bussey, 2005a), which is the ability to discriminate the familiarity of objects previously encountered (Aggleton & Brown, 1999). For example, damage to the cholinergic inputs to the PRh from the medial septum of the basal forebrain results in impaired performance on both the Novel-Object Preference (NOP) test in rats (Winters and Bussey, 2005a) and the delayed non-match-to-sample (DNMS) task in macaques (Turchi et al., 2005). ACh may influence performance on ORM tests by binding to muscarinic receptors (mAChR) in this region. For example, systemic or intra-PRh administration of mAChR antagonists, scopolamine or atropine sulfate, impairs NOP test performance in rats (Bartolini, Casamenti, & Pepeu, 1996; Ennaceur & Meliani, 1992; Huston & Aggleton, 1987; Pitsikas et al., 2001; Vannucchi, Scali, Kopf, Pepeu, & Casamenti, 1997; Tinsley et al., 2011; Warburton et al., 2003; Winters, Saksida, & Bussey, 2006) and DNMS task performance in macaques and humans (Aigner & Mishkin, 1986; Aigner, Walker, & Mishkin, 1991; Penetar & McDonough, 1983; Robbins et al., 1997; Tang & Mishkin, 1997).

MAChR binding during the familiarization phase of the NOP test appears to influence performance during the test phase. Scopolamine (0.05 mg/kg, i.p.) administered before familiarization impaired performance during the test phase (i.e. prevented novelty preference) given 15 min later (Warburton et al., 2003). Intra-PRh infusion of scopolamine (26 nM-33 mM) before familiarization also prevented novelty preference on a 15-min (Warburton et al., 2003) and 24-hr

(Winters et al., 2006) retention test. Taken together, these findings are consistent with the idea that ACh binding to mAChR in the PRh during the initial exploration of the familiar object (i.e. familiarization phase) is important for novelty preference.

When administered following familiarization, scopolamine (0.05 mg/kg, i.p.) did not influence novelty preference on a test given 15 min later (Warburton et al., 2003). However, intra-PRh scopolamine (26 nM) increased the magnitude of novelty preference when infused 0-20 hr following familiarization (Winters et al., 2006). One important difference between these two studies concerns how the novelty preference was analyzed. Within-group comparisons in the Warburton and colleagues (2003) study confirmed that both the vehicle- and scopolamine-treated rats spent more time investigating the novel relative to familiar object. A group comparison was also made, confirming no difference between the two conditions. In the Winters and colleagues (2005) study, a group comparison indicated greater proportion of time spent investigating the novel object (i.e. greater novelty preference) for the scopolamine-treated compared to vehicle-treated rats. However, no within-group comparison was conducted and so it is possible both groups demonstrated novelty preference. A lack of a within-group analysis to determine the presence of novelty preference is an important limitation of that study, as it restricts interpretations that can be drawn from the results. If both groups demonstrated novelty preference, any difference in magnitude becomes uninterpretable. Although ACh appears to bind to mAChR during familiarization to influence novelty preference, it remains to be seen

whether it also promotes novelty preference when binding to mAChR during consolidation (i.e. following familiarization).

MAChR binding enhances novelty preference on tests given between 1-60 min following familiarization (Bartolini et al., 1996; Ennaceur & Meliani, 1992; Pitsikas et al., 2001; Tang et al., 1997; Vannucchi et al., 1997). However, few studies have explored whether mAChR antagonism impairs novelty preference following delays that extend beyond 1 hr. Tinsley and colleagues (2011) reported impaired NOP test performance following systemic (0.05 mg/kg, i.p.), or intra-PRh infusion (130 nM) or scopolamine following a 20-min, but not 24-hr, retention delay. These results are consistent with the idea that mAChR binding may not influence ORM when retention delays exceed 60 min. However, additional studies using NOP test trials with retention delays longer than 60 min are necessary before making conclusions about the role of mAChR in long-term ORM.

There is evidence that mAChR binding is also important for neuronal correlates of ORM. Reduced neuronal firing or activation in the PRh is reported following exposure to familiar versus novel stimuli in macaques (Brown & Aggleton 2001) and rats (Wan et al., 2004; Wan, Aggleton, & Brown, 1999; Warburton et al., 2003, 2005; Zhu, Brown, Aggleton, 1995; Zhu, Brown, McCabe, & Aggleton, 1995). It is believed that the reduced activity serves as a neuronal basis for familiarity discrimination (Brown & Aggleton, 2001). Neuronal activation in these studies was measured via Fos expression. C-fos is an immediate early gene (IEG) and its protein, Fos, is expressed in higher quantities following

periods of increased firing. So, Fos is considered a protein marker of neuronal activity. Scopolamine (0.05 mg/kg, i.p.) administration during repeated presentation of familiar visual stimuli prevents the reduced Fos-IR in PRh neurons, suggesting that mAChR binding in this region may influence the activity patterns of PRh neurons during initial exposure to visual stimuli (Warburton et al., 2003). However, the use of visual stimuli limits the extent to which these results are informative about how these activity patterns relate to ORM, as rats are thought to obtain the majority of their sensory information from olfactory cues, (Astur et al., 2002).

Albasser, Poirier, and Aggleton (2010) addressed this limitation by examining Fos-IR following novel or familiar-object exploration. Analyses were conducted separately for the anterior (-2.76-3.84 mm from Bregma), middle (-3.84-4.8 mm from Bregma), and posterior (-4.8-6.3 mm from Bregma) portion of the PRh. Separate analyses were also conducted for Broadmann's area 35 (a35) and 36 (a36) within each portion. Consistent with previous studies that use visual stimuli, more Fos-IR cells were observed following novel- relative to familiar-object exploration. However, this difference was only observed in the posterior PRh. Taken together, studies that examine Fos-IR following stimulus presentation suggest that novel-stimulus exposure results in higher neuronal activation in the PRh. This increase may be limited to posterior regions and the degree of activation appears to be influenced by mAChR.

If reduced PRh activation serves as a neuronal basis for familiarity discrimination, preventing this activation should impair ORM. This was addressed

by Seoane, Tinsley, and Brown (2012), who demonstrated that intra-PRh infusions of antisense oligodeoxynucleotide (ODN) prevented an increase in Fos-IR in the PRh following novel compared to familiar visual stimuli presentation. Intra-PRh infusions of antisense ODN given either immediately before or after familiarization also prevented novelty preference on tests given 3 and 24 hr, but not 20 min, later. The delay-dependent effect on novelty preference combined with preventing increased visual stimulus-related PRh activation (as measured by Fos-IR) is consistent with the neuronal mechanism of familiarity discrimination proposed by Brown and Aggleton (2001). Given that mAChR is implicated in both novelty-related PRh activation and ORM, it remains to be seen whether these effects are observable in the same set of animals. It also remains to be seen whether the degree of PRh activation is related to performance on ORM tests. If such an association exists, it will provide support for the neuronal basis of familiarity discrimination.

The first goal of the present study was to determine whether mAChR is implicated in both familiarization and consolidation of object representations. Atropine sulfate (100 μM) or vehicle was infused bilaterally in the PRh prior to or immediately following the familiarization phase of the NOP test and novelty preference was measured 4 or 24 hr later. A second goal was to determine whether mAChR differentially affects novelty-related neuronal activation that occurs following in the anterior, middle, and posterior portion of a35 and a36. The same infusions were given prior to exploration of a familiar or novel set of objects. Within each infusion condition (vehicle and atropine), half the rats were

exposed to familiar object while the remaining half with novel objects. Therefore, a total of four groups was used. Atropine sulfate was chosen over the more commonly used scopolamine as it has a shorter half-life (3 instead of 4.5 hr) and would not be expected to be active during any of the retention tests.

2.2 Method and materials

2.2.1 Subjects

Twenty-eight male Long Evans rats (350-460 g; Charles River, St Constant, Quebec) were housed in pairs in transparent shoebox cages lined with a combination of woodchip and corncob bedding under 12:12 reverse light cycle (lights on at 8:00 pm) with ad libitum access to water and ~25 g daily access to chow. Following surgery, rats were housed individually for the remainder of the study. Approval for all procedures was provided by Concordia University's Animal Research Ethics Committee in accordance with the guidelines established by the Canadian Council on Animal Care.

2.2.2 Surgery

Rats were anesthetized with isoflurane gas (Jaansen, Toronto, Ontario, Canada) before receiving chronic bilateral implantation of guide cannulae (8.5 mm, 22 G, HRS Scientific) targeting the PRh (AP: -5.5 mm, ML: +/- 6.0 mm, DV: - 8 mm at 10° angle relative to vertical axis). Two rats died during surgery. A minimum recovery period of 10 days was given before behavioural testing began.

2.2.3 Drug administration

For the NOP test, rats received bilateral infusions immediately before the familiarization phase for half the trials. For the remaining half, infusions occurred

immediately after familiarization. Infusions also occurred immediately before the final object exploration session. In all instances, rats received infusions of either atropine sulfate (100μM, 1.2 μl/side) dissolved in artificial cerebral spinal fluid (aCSF; 145.11 mM NaCl, 2.68 mM KCl, 2.13 mM $MgCl_2$, 1.96 mM Na_2HPO_4, 0.905 mM $CaCl_2*2H_2O$, 0.199 mM ascorbic acid), or vehicle (aCSF, 1.2 μl/side).

2.2.4 Apparatuses

2.2.4.1 NOP test. An open field arena (60 cm X 70 cm X 70 cm) constructed of grey PVC plastic was used. The flooring was made of stainless steel and was covered with woodchip bedding. A video camera was positioned above the arena and both familiarization and test phases of each trial were recorded to behavioral analysis.

A total of six objects (one pair per trial) served as test stimuli and were made of porcelain, ceramic, metal, glass, or plastic, and varied 5-15 cm, in height. Three copies of each object were used, and objects serving as novel stimuli were counterbalanced across rats in each condition. Each pair had been previously been screened to ensure that both objects evoked similar amounts of investigation in naïve rats. Objects were affixed to the bottom of glass jars, and attached to the floor of the apparatus by screwing the jars into lids fixed in place. The objects were positioned 27 cm from opposing corners of the arena.

2.2.4.2 Neuronal activation following object exploration. A circular open field with grey PVC flooring (100 cm X 100 cm), and walls constructed from black fiberglass was used. Ten objects with similar characteristics as those used in NOP test were used, five of which served as the familiar stimuli, whereas the

remaining 5 served as novel stimuli. Each object was fixed to a glass jar, and inverted jar lids were attached to a small aluminum plate, approximately 4 cm x 10 cm. Two small pins attached at each end of the aluminum plate extended downward 2.5 cm, and fit inside small holes, serving to fix the objects in place. Similar to the open field used for the NOP test, a video camera was positioned above the circular arena to allow for behavioural analysis.

2.2.5 Procedures

A total of 3 NOP test trials were given. Prior to the first test, rats received two 5-min habituation sessions to the open field with 2 identical copies of an object. Twenty-four hours later, rats received infusions of atropine (n = 13) or vehicle (n = 13) before being re-introduced to the open field with 2 copies of another object (i.e. sample objects) for the 5-min familiarization phase. Twenty-four hours later, rats returned to the apparatus for the 5-min retention test, whereby a third copy of the sample object and a novel object were presented. The second NOP test trial was administered in the same manner, but a shorter (i.e. 4 hr) retention delay was used. The final trial involved a 24-hr retention delay much like the first trial, but infusions occurred following approximately 10 min following the familiarization phase.

After all NOP test trials were given, rats were exposed to five additional objects in the circular open field for two 7-min sessions spaced 24 hr apart. Twenty-four hours after the second session, rats received bilateral infusions of either atropine sulfate or vehicle before being exposed to the same 5 objects

(Familiar-Atropine: *n* = 5; Familiar-Vehicle: *n* = 6), or to 5 novel objects (Novel-Atropine: *n* = 6; Novel-Vehicle: *n* = 6).

2.2.6 Histology and immunohistochemistry

Approximately 60 min following the final exploration session, rats received a lethal dose of sodium pentobarbital followed by transcardial perfusions with 0.9% saline (250 ml) followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (pH = 7.4, 250 ml). The brains were excised and stored in 4% paraformaldehyde solution for 4 hr before being transferred to 30% sucrose/water solution overnight. The next day, the brains were transferred to a -80°C freezer until sectioning. Using a cryostat microtome, three sets of 40 µm coronal sections were collected starting at approximately 3.2 mm anterior to Bregma until 7.62 mm posterior to Bregma. One additional set of sections through the PRh was mounted on glass microscope slides, and Cresyl violet staining was performed on the tissue for histological analysis. The other set of sections were stored in Watson's Cryoprotectant solution and stored in -20°C freezer.

On the first day of immunohistochemistry, sections were rinsed in Tris-buffered saline (TBS; Sigma-Aldrich Canada, Ltd., Oakville, ON, Canada) three times for 5 min each. The sections were then transferred to a solution containing TBS and 30% hydrogen peroxide, then incubated for 30 min at 4°C. A second set of 3 washes was given followed by 90 min incubation in a solution of TBS, 3% normal goat serum and 0.3% Triton X-100. Following a third set of washes in TBS, the sections were incubated in a rabbit polyclonal anti-Fos (1:10000,

Sigma-Aldrich) solution for 48 hr at 4°C. The second day began with a set of TBS washes followed by incubation in a solution containing a secondary antibody (biotinylated anti-rabbit Ig G) at 4°C for 60 min. This was followed be another set of washes in TBS, then processed in an avidin-biotin complex solution (ABC Elite kit, Vector Laboratories, Canada) for 60 min at 4°C. Following the final set of TBS washes, the sections were immersed in diamino-benzidine-2,3 (DAB) using a DAB-peroxidase kit (Vector Laboratories, Canada), which provides a nickel stain to permit visualization of Fos expression. Sections were stored in TBS until they were mounted to gel-coated microscope slides and cover slipped for microscopic evaluation.

2.2.7 Statistical and image analysis

Results are expressed as mean (+/- SEM) in *Figures* 2-4. All statistical analyses were conducted using SPSS version 22 for Windows (IBM, Chicago, IL) and type I error rate was set at $\alpha = .05$.

2.2.7.1 NOP test

Time spent investigating each object during familiarization and test phases was scored using ODLog version 2.7.2 for Windows (Macropod, software). A rat was considered to be investigating an object when rearing with at least one forepaw touching it, or when her head was within 3 cm of the stimulus. Biting, climbing, and sitting on the object were not considered object investigation. An investigation ratio for the first 2 min of the test phase for each trial was calculated as the proportion of total investigation time spent exploring the novel object ($time_{novel}/(time_{novel} + time_{familiar}$). These ratios were averaged separately by

infusion type under each retention delay. One-sample t-tests comparing the investigation ratios under each infusion condition for each NOP test trial was compared to chance level (0.5). Two mixed-factorial ANOVAs were also conducted. One was run on the investigation ratios obtained during the two pre-familiarization infusion trials with infusion condition as the between-subjects factor and retention delay as the within-subjects factor. Another mixed-factorial ANOVA was also conducted on the time spent investigation objects during the familiarization phase of these trials. One-way ANOVAs were conducted on the post-familiarization infusion trial. One ANOVA was conducted on the preference investigation ratios obtained during the test, and another was run on the time spent investigating objects during the familiarization phase. Pearson product-moment correlation coefficients were obtained between the time spent investigating during familiarization and the investigation ratio obtained during the test. Coefficients were calculated separately for each of the three NOP test trials.

2.2.7.2 Fos analysis

Tissue was analyzed using a Hitachi 3CCD camera (Model # HV-C20) mounted on a Leica DMR-HC microscope. Images of PRh tissue were taken on a Macintosh G4 computer using Scion Image software 1.66 at 40x magnification. Separate images were obtained for a35 and a36 in the anterior (2.56-4.16 mm from Bregma), middle (4.30-6.04 mm from Bregma), and posterior (6.30-6.72 mm from Bregma) portion of the PRh. The borders of these two sub-regions are based on those outlined by Burwell (2001). Using Image J software (http://rsb.info.nih.gov/ij/), a sampling section (250 μm X 250 μm) was selected

from each image and the number of c-Fos positive cells was counted. The experimenter was blind to group membership. Averages were calculated for each rat for each of the six subsections of the PRh (area 35 and 36 in the anterior, middle and posterior PRh). A 2X2 between-groups ANOVA was conducted with infusion condition as one factor and object exploration as the second factor for each subsection. Statistically significant interactions were followed-up with planned comparisons comparing the Novel-Vehicle group to the Familiar-Vehicle group, the Novel-Atropine group to the Novel-Vehicle group, and the Novel-Atropine group to the Familiar-Vehicle group.

Pearson product-moment correlation coefficients between investigation ratios obtained during the NOP test trials and amount of Fos-IR within each portion of each sub-region were also calculated.

2.3 Results

2.3.1 Histology

Histological analysis indicated incorrect cannula placement for 7 rats and so data from these animals were removed from all analyses. *Figure 2.1* presents the location of cannula tips in each hemisphere for all rats included in the study. A photomicrograph of a representative section is shown in *Figure 2.2* and indicates the extent of damage that occurred due to cannula placement and multiple injections.

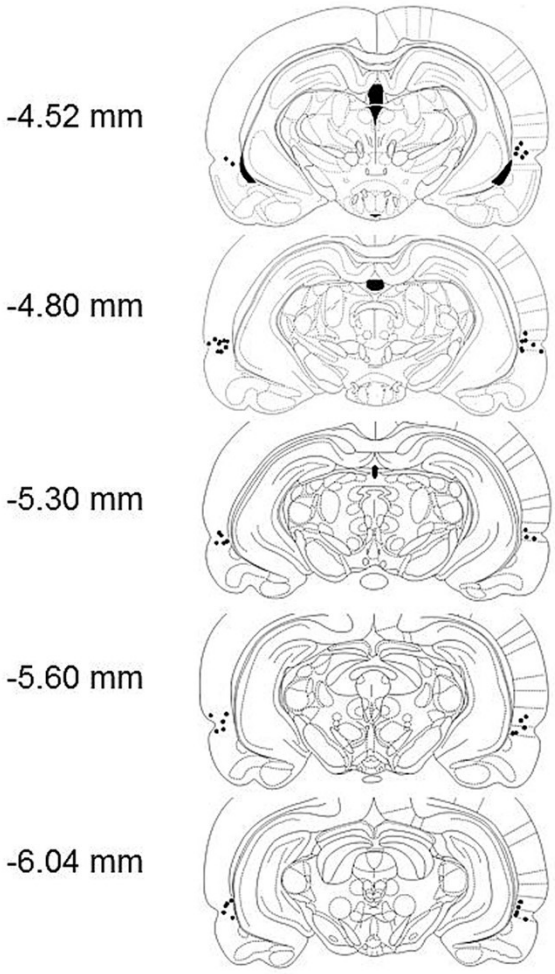

Figure 2.1: Illustration of placement of cannula tips (indicated with black circles) in both hemispheres of all rats. Some tips are presented in multiple sections. Values indicate distance from Bregma (Paxinos & Watson, 1998).

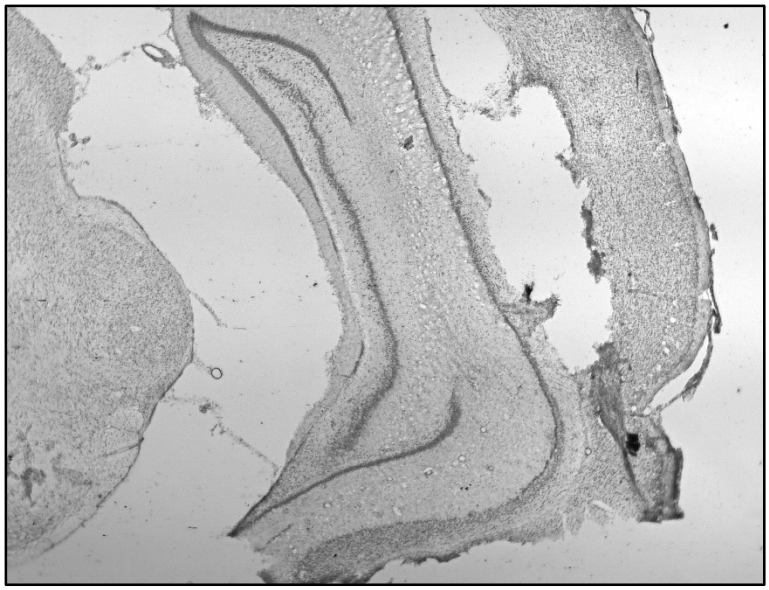

Figure 2.2: Photomicrograph of stained tissue depicting placement of a guide cannula in the PRh with some damage from injector and multiple infusions.

2.3.2 NOP test

2.3.2.1 Pre-familiarization infusion trials. Figure *2.3A* presents the total time spent investigating objects during the 5-min familiarization phase for the two NOP test trials. Object investigation is presented separately by infusion group (vehicle and atropine). A mixed factorial analysis of variance (ANOVA) was conducted and revealed no statistically significant effect of retention delay, $F(1, 17) = 1.25$, $p = .28$, infusion condition, $F(1, 17) = 0.02$, $p = .90$, partial $\eta^2 = .001$, or interaction, $F(1, 17) = 0.08$, $p = .79$, partial $\eta^2 = .004$.

Figure 2.3B presents the investigation ratios for each group at each retention delay. One-sample t-tests (one-tailed) were conducted to compare performance to chance (0.5). The vehicle group demonstrated above-chance preferences following both retention delays, 4 hr: $t(11) = 4.25$, $p = .000$, Hedge's g = 1.23, 24 hr: $t(11) = 1.87$, $p = .04$, Hedge's g = 0.54. The atropine group demonstrated no preference at either delay, 4 hr: $t(6) = 1.61$, $p = .079$, Hedge's g = 0.61, 24 hr: $t(6) = -0.28$, $p = .393$, Hedge's g = -0.11. A mixed factorial ANOVA was conducted on the investigation ratios and a statistically significant main effect of infusion condition was found, $F(1, 17) = 4.77$, $p = .043$, partial $\eta^2 = .22$. No main effect of delay, $F(1, 17) = 2.44$, $p = .14$, partial $\eta^2 = .13$, and no delay by infusion interaction, $F(1, 17) = 0.07$, $p = .79$, partial $\eta^2 = .004$, was found.

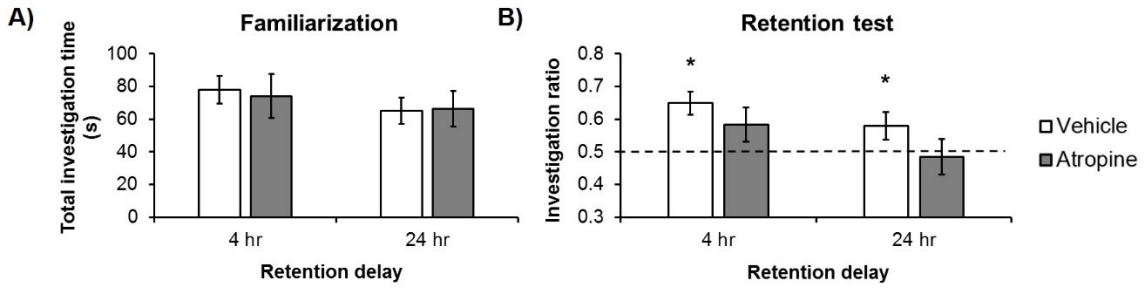

Figure 2.3: Performance on pre-familiarization infusion trials of NOP test. A. Mean time spent investigating the sample object for both groups during the 5-min familiarization phase prior to the 4- and 24-hr retention delay. B. Investigation ratios of both groups obtained during the first 2 min of the 4- and 24-hr retention test. (*p < .001-.05; one-sample t-test).

The total time spent investigating objects during the test phase was also analyzed using a mixed factorial ANOVA. A statistically significant main effect of retention delay was found, $F(1, 17) = 4.83$, $p = .04$, partial $\eta^2 = .22$. No significant main effect of infusion condition, $F(1, 17) = 0.14$, $p = .71$, partial $\eta^2 = .01$, and no infusion by delay interaction was found, $F(1, 17) = 2.99$, $p = .10$, partial $\eta^2 = .15$.

2.3.2.2 Post-familiarization infusion trials. *Figure 2.4A* presents the time spent investigating objects during the familiarization phase of the post-familiarization infusion trial of the NOP test. A one-way between-groups ANOVA revealed no statistically significant group differences, $F(1, 17) = 0.99$, $p = .33$, $\eta^2 = .06$. *Figure 2.4B* presents the investigation ratios obtained for each trial type. One-sample t-tests (one-tailed) were conducted separately by group. An above-chance preference was found for the vehicle group, $t(11) = 2.19$, $p = .026$, Hedge's g = 0.63, but not the atropine group, $t(6) = 0.82$, $p = .221$, Hedge's g = 0.31. One-way between-groups ANOVAs were conducted on the investigation ratio and time spent investigating objects during the test phase. No group differences were found, ratio: $F(1, 17) = 0.57$, $p = .462$, $\eta^2 = .03$, time: $F(1, 17) = 1.22$, $p = .29$, $\eta^2 = .07$.

2.3.2.3 Correlations between NOP test performance and object investigation during familiarization. Pearson correlations were conducted on investigation time during familiarization and the investigation ratio during the test of each NOP trial. None of the correlations were statistically significant, r = -.39-.08, p = .097-.98.

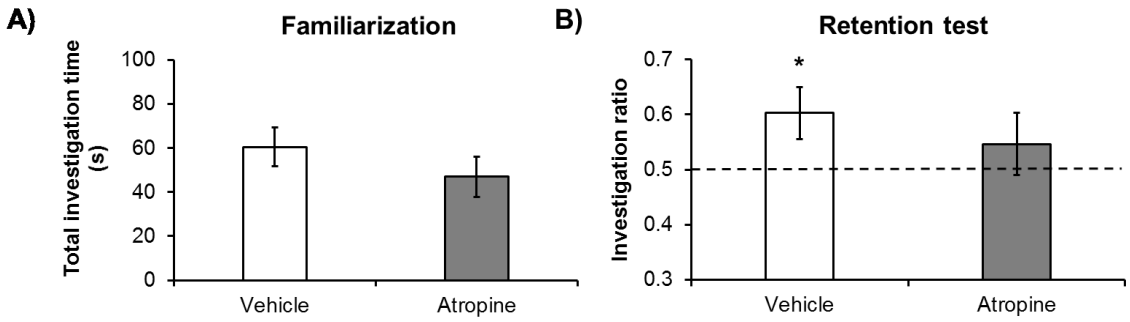

Figure 2.4: Performance on post-familiarization infusion trials of NOP test. A. Mean time spent investigating the sample object for both groups during the 5-min familiarization phase prior to the 24-hr retention delay. B. Investigation ratios of both groups obtained during the first 2 min of retention test. (*$p < .05$; one-sample t-test).

2.3.3 Fos expression

A two-way between-groups ANOVA was conducted on the number of Fos-IR cells in the samples obtained from each of the 6 subsections (a35 and a36 in the anterior, middle, and posterior) of the PRh. The first factor was infusion condition (vehicle and atropine) and the second factor was the object exploration during the final session (familiar and novel). A statistically significant interaction between the two factors was found for the posterior sub-region of a36, $F(1, 11) = 11.79$, $p = .006$, $\eta^2 = .52$. Neither of the main effects were statistically significant, Infusion condition: $F(1, 11) = 1.59$, $p = .233$, $\eta^2 = .13$, Object exploration: $F(1, 11) = 0.01$, $p = .925$, $\eta^2 = .001$. Planned comparisons revealed statistically significantly more Fos-IR cells in the Novel-Vehicle condition than both the Familiar-Vehicle, $t(8) = 2.50$, $p = .037$, Hedge's g = 1.61, and Novel-Atropine groups, $t(7) = 3.84$, $p = .006$, Hedge's g = 2.72. There were no differences in the number of Fos-IR cells in the Familiar-Vehicle and Novel-Atropine groups, $t(5) = 1.07$, $p = .334$, Hedge's g = 0.81. *Figure 2.5* presents photomicrographs of representative sections of the posterior sub-region of a36, illustrating the extent of Fos-IR in each group.

No statistically significant main effects or interactions were observed for remaining sub-regions of a36, and for all sub-regions of a35, Infusion condition: $F(1, 12) = 0.04\text{-}2.00$, $p = .185\text{-}.855$, $\eta^2 = .003\text{-}.15$, Object exploration:

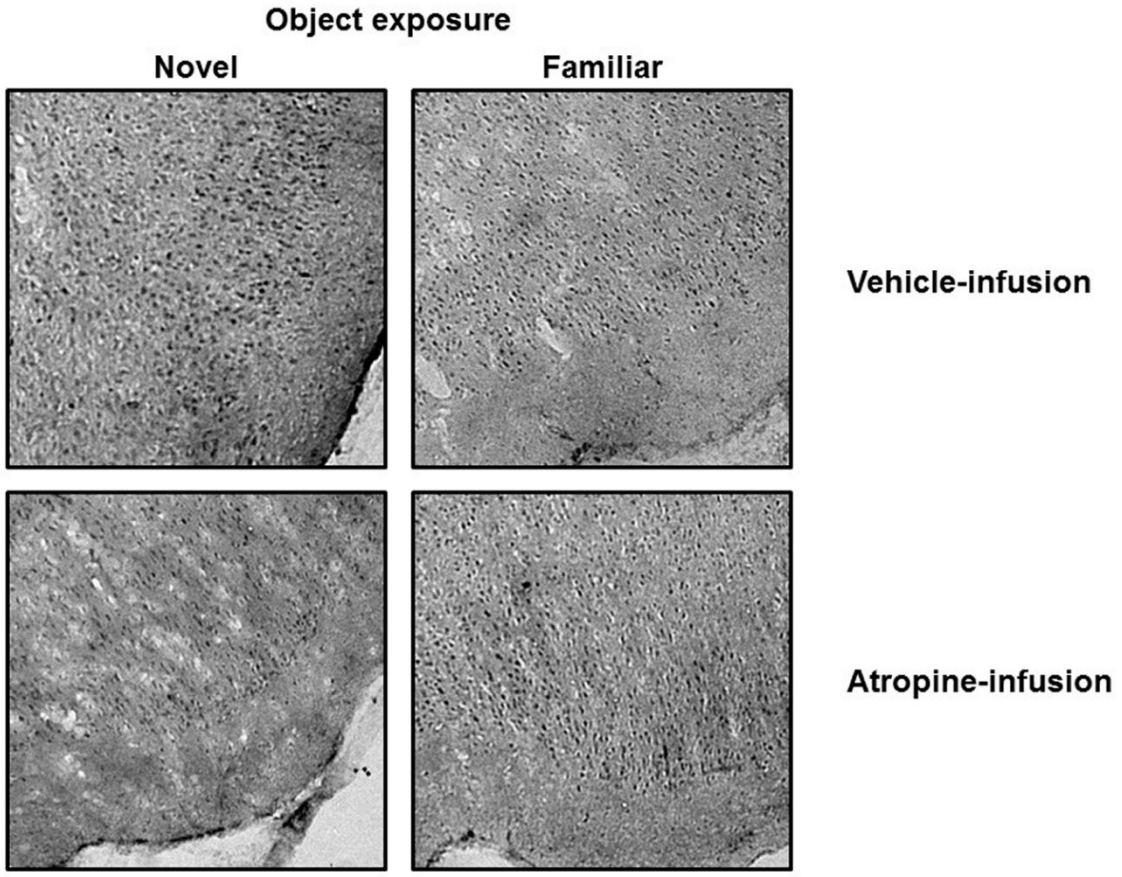

Figure 2.5: Representation of Fos-IR cells in the PRh. Photomicrographs depicting Fos-IR cells in the posterior portion (-6.3-6.72 mm from Bregma) of a36 taken at 10x magnification. The top panel (A-B) shows representative sections from rats that were infused with vehicle, while the bottom panel (C-D) shows sections from those infused with atropine sulfate (100 μM). The left panel (A & C) depicts sections from those presented with novel objects on the final exploration day while the right panel (B & D) presents sections from rats exposed to familiar objects.

$F(1, 12) = 0.00\text{-}4.53$, $p = .055\text{-}.996$, $\eta^2 = .00\text{-}.27$, and interaction: $F(1, 12) = 0.003\text{-}1.67$, $p = .22\text{-}.958$, $\eta^2 = .00\text{-}12$. *Figure 2.6* presents the number of Fos-IR cells in each sub-region of a35 and a36.

2.3.4 Correlation between NOP test performance and Fos-IR

Pearson correlations were calculated on performance on the NOP test and the number of Fos-IR cells. Separate correlations were conducted for each subsection of a35 and a36, and also for each NOP test trial. Statistically significant correlations were found between the number of Fos-IR cells in the anterior portion of a36 and performance on the post-familiarization infusion NOP test trial, $r = -.70$, $p = .017$, and also between the middle portion of a36 and performance on the pre-familiarization infusion trial following a 24-hr retention delay, $r = -.67$, $p = .024$. All other correlations were not statistically significant, $r = .02\text{-}.57$, $p = .07\text{-}.95$.

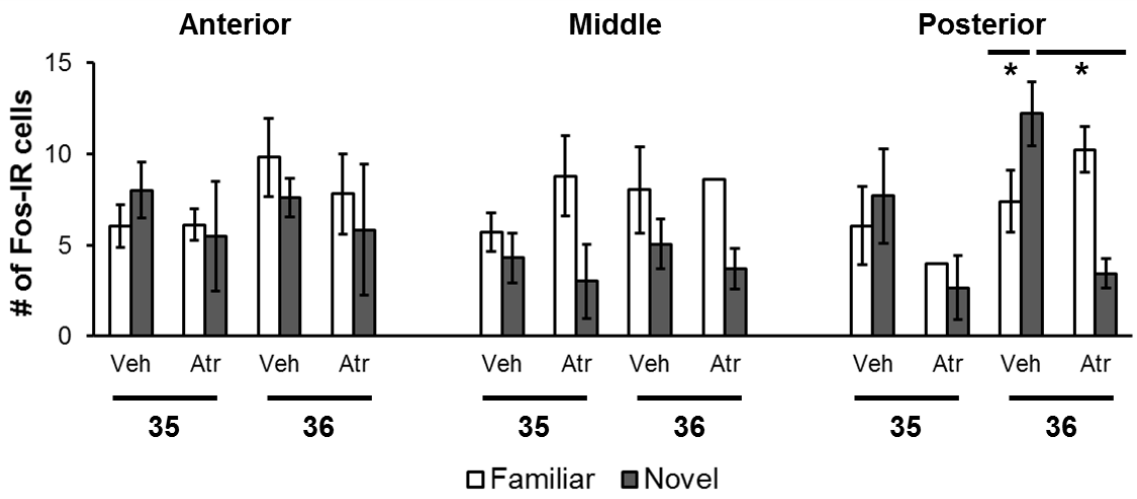

Figure 2.6: Mean number of Fos-IR cells in each portion (anterior, middle, posterior) of a35 and a36 for each group. (*p < .05; independent-samples t-test). *Note.* Veh = Vehicle; Atr = Atropine.

2.4 Discussion

The results from the NOP test suggest that intra-PRh infusion of atropine sulfate (100 μM), a mAChR antagonist, prevents novelty preference regardless of retention delay and timing of infusions. While the vehicle-treated group demonstrated novelty preference on all three NOP test trials, the atropine-treated group did not. These results support a role of mAChR in the PRh in novelty preference. The role appears to be consistent across retention delays.

On the pre-familiarization infusion trials, between-group comparisons were conducted and revealed lower novelty preference in the atropine- relative to vehicle-treated group. In addition to preventing novelty preference following both retention delays, intra-PRh infusion of atropine also lowered the magnitude of the preference ratio. The within- and between-group comparisons are consistent with both Warburton and colleagues (2003) and Winters and colleagues (2006) and together, support the idea that ACh binds to mAChR during familiarization to enhance novelty preference on retention tests given up to 24 hr later. This is consistent with studies demonstrating impaired ORM in macaques following intra-PRh infusion of scopolamine prior to the DNMS task (Tang et al., 1997). The results are also consistent with a general role of ACh in the acquisition of new information (Hasselmo, 2006).

The present study demonstrates an effect of mAChR antagonism on novelty preference following retention delays (4 & 24 hr) that are longer than those used in the majority of previous studies (≤ 1 hr; Bartolini et al., 1996; Ennaceur & Meliani, 1992; Pitsikas et al., 2001; Tinsley et al., 2011; Vannucchi et

al., 1997; Warburton et al., 2003). This is an important strength of the study, as it suggests the role of mAChR may extend to long-term memory.

The lack of a delay-dependent effect on novelty preference in the present study is consistent with an effect of mAChR antagonism on non-cognitive processes that influence the expression of novelty preference. Tang and colleagues (1997) reported lower accuracy on DNMS task performance following intra-PRh infusion of scopolamine. However, the authors of that study did not compare performance across several retention delays. Thus, it is possible those impairments are also the result of non-cognitive processes. Alternatively, the chosen retention delays in the present study may have been ineffective at demonstrating a delay-dependent effect on novelty preference. Tinsley and colleagues (2011), who reported delay-dependent effects of intra-PRh scopolamine infusion on novelty preference, used a much shorter delay of 20-min than the shortest delay used in the present study (4 hr). However, this explanation is unlikely as Warburton and colleagues (2003) reported a failure to demonstrate novelty preference on a 15-min retention test following systemic and intra-PRh infusion of scopolamine. While the results from the pre-familiarization trials of the present study support a role of ACh (via mAChR in the PRh) in novelty preference, the lack of a delay-dependent effect does not provide convincing support for a role in ORM.

Intra-PRh infusions of atropine given immediately following familiarization also prevent novelty preference on a test given 24 hr later. This result suggests that the role of ACh (via mAChR) in novelty preference may not be limited to the

familiarization phase, but also extends to the consolidation phase. This is inconsistent with results reported by Warburton and colleagues (2003) and Winters and colleagues (2006). Warburton and colleagues (2003) reported no effect of post-familiarization antagonism of mAChR on novelty preference observed on a test given 15-min later. Winters and colleagues (2006) reported greater novelty preference following intra-PRh scopolamine infusion relative to vehicle infusion. Winters and colleagues (2006) interpreted the group difference as a result of retroactive interference. Specifically, they suggested that the infusion episodes interfered with encoding of object representations and that intra-PRh scopolamine infusions blocked this interference, resulting in intact ORM. In the present study, intact novelty preference was observed in the vehicle-treated group, which doesn't support an interfering effect of the infusion episode on novelty preference. Since no within-group comparisons were conducted in the Winters and colleagues (2006) study, it is possible that novelty preference was intact in both groups and that mAChR antagonism increased the magnitude of the preference while having no effect on ORM. In the present study, the failure of the atropine-treated group to demonstrate novelty preference on the post-familiarization infusion trial is consistent with an effect of ACh via mAChR on the consolidation of object representations. However, it is also consistent with an effect on non-cognitive processes. This is particularly important, as the results from the pre-familiarization infusion trials are also consistent with an effect on non-cognitive processes. Given that the present set

of findings cannot clarify the nature of the effect of mAChR antagonism on ORM, future studies are needed to disambiguate the role of these receptors.

In addition to preventing novelty preference, intra-PRh antagonism of mAChR also influences novelty-related neuronal activation. Within the vehicle-treated condition, the number of Fos-IR neurons was higher following novel- relative to familiar-object exploration. This difference was observed in the posterior portion of a36 and not in any portion of a35. More Fos-IR cells following novel- relative to familiar-object exploration is consistent with previous research (Wan et al., 1999, 2004; Warburton et al., 2003, 2005; Zhu, Brown, McCabe, et al., 1995), including the study by Albasser and colleagues (2010), which reports differences in the posterior portion of the PRh (although they reported group differences in Fos-IR in both a35 and a36). MAChR antagonism prior to novel-object exploration prevented an increase in neuronal activity, as the number of Fos-IR cells in the Novel-Atropine group was lower than in the Novel-Vehicle group, and similar to the Familiar-Vehicle group. This effect does not appear to be due to a non-specific effect of atropine on neuronal activation, as there was no main effect of infusion condition on Fos-IR. The present study along with the one by Warburton and colleagues (2003) suggests ACh influences novelty-related neuronal activation via mAChR.

Should a reduction in neuronal firing of PRh neurons serve as a neuronal basis for familiarity discrimination, as proposed by Brown and Aggleton (2001), the number of Fos-IR cells in this region would be related to performance on a measure of ORM. The study by Seoane and colleagues (2012), which

demonstrates that blocking c-fos mRNA expression (via fos antisense ODN) prevents novelty preference, is certainly consistent with this prediction. However, no positive correlations were found between Fos-IR in the PRh and performance on the NOP test trials of the present study. Albasser and colleagues (2010) also report no association between Fos-IR in the PRh and performance on the NOP test. The failure to establish a relationship between the two is certainly consistent with a lack of association between neuronal activation in the PRh and novelty preference. However, it may also be that a non-linear association exists. For instance, perhaps there is a minimal amount of neuronal activation required for novelty preference to occur, after which any additional increase in activation does not further influence novelty preference.

It is also possible that the lack of correlation is the result of novelty preference being a poor indicator of the strength of ORM ability rather than reflecting a lack of association between PRh activation and ORM. Gaskin and colleagues (2010) demonstrate that investigation ratios higher in magnitude do not reflect superior ORM (Gaskin et al., 2010). Therefore, an association between PRh activation and ORM may not be evident when using preference ratios as a measure of this ability. Instead, accuracy scores on the nonrecurring-items DNMS task should be used, as they reflect the strength of ORM. Examining the association between novel-object exploration-related PRh activation and DNMS task performance is an important next step.

A third possibility for the lack of association between NOP test performance and PRh activation is that c-fos is not sensitive to learning-specific

neuronal activation in the PRh. Guzowski, Setlow, Wagner, and McGaugh (2001) conducted a study comparing the expression of activity-regulated cytoskeletal-associated (Arc) gene to both c-fos and zif268 following a learning episode. Although all three are IEGs, c-fos and zif268 are classified as regulatory transcription factors that have non-specific effects on neuronal function, whereas Arc influences the expression of "effector" proteins that modulate specific neuronal functions. Following learning, expression of these IEGs were positively correlated with one another within a structure, although expression of Arc was the only one to also correlate positively with performance on the learning task (Guzowski et al., 2001). Although c-fos may play a role in novelty preference, as demonstrated by Seoane and colleagues (2012), it is possible the expression of other IEGs, including Arc, is more strongly associated with novelty preference and/or ORM.

The results of the present study further our understanding of the role of mAChR in the PRh in novelty preference, but do not provide convincing evidence for a role in ORM. The present study used object stimuli and not simply visual stimuli to demonstrate that the posterior portion of a36 (but not a35) is involved in novelty-related neuronal activation, and that mAChR plays a role in this activation. Although no association was found between novelty preference and Fos expression, the results do provide some support for the proposed neuronal mechanism of familiarity discrimination. More research involving different ORM tests and IEGs is necessary before concluding that no association exists.

CHAPTER 3

Systemic and intra-rhinal-cortical 17-β estradiol administration modulate object-recognition memory in ovariectomized female rats

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Preface

The previous chapter provides evidence that ACh modulates novelty preference and novelty-related neuronal activation in the posterior a36 via muscarinic receptors. In females, estrogens, including E2, are implicated in cognitive ability of humans, non-human primates, and rodents, with evidence suggesting higher levels of E2 are associated with improved ORM. Therefore, E2 appears to be another modulator of ORM. Since the majority of studies conducted in rodents involve the NOP test as the sole measure of ORM, it remains to be seen whether the enhancing effect of E2 is specific to novelty preference, or whether the effect extends to ORM. One goal of the present study was to determine whether E2 improves performance on both the NOP test and DNMS task.

While most studies compare ovariectomized females with and without E2 replacement, it remains to be seen whether the pattern of results changes when the comparison group experiences physiologically-relevant, low levels of E2. Thus, an additional goal was to compare a chronic low E2 plus acute high E replacement condition to a chronic low E2 replacement condition.

Finally, it is currently unknown whether E2 modulates ORM by influencing PRh function and so the third goal was to determine whether E2 acts within the PRh to modulate this ability. OVX female rats were tested on the NOP test (Experiment 1) and the DNMS task (Experiment 2) under several hormonal conditions (acute high E replacement vs chronic low E; and intra-PRh/EC infusion of E2 vs vehicle).

The main findings were that systemic and intra-PRh/EC E2 enhanced novelty preference, but impaired DNMS task performance in a delay-dependent manner. These divergent effects contradict previous conclusions that were made based on effects of E2 replacement on the NOP test performance. Instead, they suggest that higher E2 levels are not associated with improved ORM abilities, and that any treatment effect on novelty preference should be interpreted with caution.

Abstract

Previous studies using the novel-object-preference (NOP) test suggest that estrogen (E) replacement in ovariectomized rodents can lead to enhanced novelty preference. The present study aimed to determine: 1) whether the effect of E on NOP performance is the result of enhanced preference for novelty, *per se*, or facilitated object-recognition memory, and 2) whether E affects NOP performance through actions it has within the perirhinal cortex/entorhinal cortex region (PRh/EC). Ovariectomized rats received either systemic chronic low 17-β estradiol (E2; ~20 pg/ml serum) replacement alone or in combination with systemic acute high administration of estradiol benzoate (EB; 10 µg), or in combination with intracranial infusions of E2 (244.8 pg/µl) or vehicle into the PRh/EC. For one of the intracranial experiments, E2 was infused either immediately before, immediately after, or 2 hr following the familiarization (i.e., learning) phase of the NOP test. In light of recent evidence that raises questions about the internal validity of the NOP test as a method of indexing object-recognition memory, we also tested rats on a delayed nonmatch-to-sample (DNMS) task of object recognition following systemic and intra-PRh/EC infusions of E2. Both systemic acute and intra-PRh/EC infusions of E enhanced novelty preference, but only when administered either before or immediately following familiarization. In contrast, high E (both systemic acute and intra-PRh/EC) impaired performance on the DNMS task. The findings suggest that while E2 in the PRh/EC can enhance novelty preference, this effect is probably not due to an improvement in object-recognition abilities.

3.1 Introduction

Estrogens (E), including the most common and potent form, 17-β estradiol (E2), are proposed neuromodulators of learning and memory. The most compelling evidence comes from studies examining rats' performance on behavioural tasks that require different learning and memory abilities. Although the majority of research demonstrates a modulatory role, inconsistency remains as to whether E enhances or impairs these abilities. A number of factors may influence the direction of results, including differences in type of E being administered, dose, interactions with other hormones, duration of treatment, mode of delivery, sex, stress, motivation, and the specific memory abilities that are required for task performance (Korol, 2004; Zurkovsky, Brown, Boyd, Fell, & Korol, 2007).

High E2 replacement (rats: 5-30μg/kg acute; mice: 0.2-0.4 mg/kg, acute, s.c.; 1-2.5 μM, chronic, p.o.) to ovariectomized (OVX) rodents is also associated with improved object-recognition memory (ORM; Fernandez & Frick, 2004; Gresack & Frick, 2006; Inagaki, Gautreaux, & Luine, 2010; Luine, Jacome, & MacLusky, 2003), which is the ability to discriminate the familiarity of objects that have been previously encountered (Aggleton & Brown, 1999). In rodents, the effect of E2 on ORM has been examined exclusively using the *novel-object-preference* (NOP) test, which measures novelty preference. OVX rats with systemic high acute E2 replacement (15 μg/kg) demonstrate novelty preference on a test 4 hr later whereas OVX rats without E2 replacement do not (Luine et al., 2003). High acute E replacement (15 μg/kg of diethylstilbestrol and 30 μg/kg

E2) given immediately, but not 2 hr following familiarization produces comparable results, suggesting E enhances consolidation (Luine et al., 2003). These and other studies (Fernandez & Frick, 2004; Gresack & Frick, 2006; Inagaki et al., 2010) provide evidence that E enhances novelty preference.

Another test of object-recognition is the delayed nonmatch-to-sample (DNMS) task (e.g. Aggleton, 1985; Kesner, 1991; Mumby, Pinel, & Wood, 1990; Rothblat & Hayes, 1987). Studies using human and non-human primates suggest OVX impairs, while OVX with E replacement improves performance on the DNMS and delayed matching-to-sample tasks (Craig et al., 2010; Lacreuse, Herndon, & Moss, 2000; Rapp, Morrison, & Roberts, 2003; Voytko, Higgs, & Murray, 2008), although some have reported no effect (Lacreuse & Herndon, 2003). Currently, no study has examined the effect of E on DNMS performance in rats.

Increasingly, evidence suggests that the perirhinal cortex (PRh) is necessary for object-recognition memory. Damage to the PRh, either alone or in combination with entorhinal cortex (EC), impairs performance on the DNMS task (Mumby & Pinel, 1994; Wiig & Bilkey, 1995) and NOP test (Bussey, Muir, & Aggleton, 1999; Bussey, Duck, Muir, & Aggleton, 2000; Ennaceur, Neave, & Aggleton, 1996; Mumby, Glenn, Nesbitt, & Kyriazis, 2002; Mumby, Piterkin, Lecluse, & Lehmann, 2007; Winters, Forwood, Cowell, Saksida, & Bussey, 2004). Although a few studies reported impairments following lesions of the hippocampus (HPC; Clark, Zola, & Squire, 2000; Clark, West, Zola, & Squire, 2001), most find no such evidence (for a review see Mumby, 2001), and so

controversy remains as to the role played by the HPC in ORM (Barker & Warburton, 2011; Winters, Saksida, & Bussey, 2008). Studies examining how E modulates novelty preference have focused exclusively on the HPC. Given the inconsistencies in the literature on the role of the HPC in object recognition and the limited number of studies to date on the effects of E, it appears premature to conclude E acts only within the HPC to modulate object-recognition memory. ERs are expressed in both PRh and EC (Blurton-Jones & Tuszynski, 2002; Shima, Yamaguchi, & Yuri, 2003; Shrughrue, Lane, & Merchentaler, 1997, 2001), and so it is possible that E may act in these structures to modulate this ability.

The present study investigated whether E modulates performance on both the NOP test and DNMS task, and if so, whether this can be explained by its effects in the PRh/EC. Although previous studies compared OVX rats under high acute E2 replacement to those without replacement, the present study compared high acute E replacement to chronic low E2 replacement. In Experiment 1A, OVX rats with chronic low E2 replacement (~20 pg/ml serum) were administered systemic high acute E replacement (β-estradiol benzoate, EB; 10 μg) or oil vehicle prior to the familiarization phase of each NOP trial. High acute E replacement was hypothesized to improve object-recognition memory, and so rats were expected to demonstrate enhanced novelty preference following EB administration relative to the rats administered oil that were already receiving chronic low E replacement. To the extent that enhanced novelty preference reflects enhanced objects-recognition memory, an improvement of this ability by high E may be due to modulation of processes that occur during familiarization,

when features of the sample object are encoded. It may also be due to modulation of processes occurring during the minutes and hours following familiarization, when the encoded information is undergoing consolidation. Experiment 1B was designed to examine whether intra-rhinal-cortical (PRh/EC) E2 modulates encoding and/or consolidation. OVX rats (with chronic low E2 replacement) received bilateral intracranial infusions of E2 (244.8 pg/µl per side) and vehicle either immediately before, immediately after, or 2 hr following familiarization. The infusion concentration of E2 used in the present study is twice as high as that used for intra-HPC infusions (Packard & Teather, 1997b; Zurkovsky et al., 2007).

Experiment 2 tested the effects of E on DNMS task performance. In Experiment 2A, rats that had been trained to perform a DNMS task received systemic injections of EB or oil with chronic low E2 on board. Experiment 2B assessed the effects of intra-rhinal-cortical infusion of E2 on DNMS task performance. If E is associated with improved object-recognition memory, then higher accuracy should be observed following high acute E replacement (in Experiment 2A) and intracranial infusions of E2 (in Experiment 2B). The effect of E on DNMS task performance should be more observable following longer retention intervals than shorter ones.

3.2 Experiment 1

3.2.1 Material and methods

3.2.1.1 Subjects

Twenty-seven female Long Evans rats (Charles River, Saint-Constant, Quebec) weighing between 175-200g at time of arrival served as subjects. Rats were housed in pairs in transparent shoebox cages lined with a combination of woodchip and corncob bedding under 12:12 reverse light cycle (i.e. lights on at 8:00pm) with *ad libitum* access to water and ~25g daily access to chow. For Experiments 1B, rats were housed individually following surgery for the remainder of the study. Approval for all procedures was provided by Concordia University's Animal Research Ethics Committee in accordance with the guidelines established by the Canadian Council on Animal Care.

3.2.1.2 Surgery

Following approximately two weeks of acclimation to the housing conditions, all rats were anesthetized with isoflurane (Jaansen, Toronto, Ontario, Canada) and bilateral ovariectomies were performed using a standard aseptic procedure through a lumbar incision. In addition, 1 cm silastic tubing (i.d. 1.47 mm; o.d. 1.96 mm) was implanted at the nape of the neck. The implants were replaced every four weeks (8 times total), and a delay of 7 days occurred between implantation and data collection. This was to ensure circulating low E2 levels were stable throughout the 21-day period during which time behavioural testing occurred.

Rats in Experiments 1B (N = 8) were subsequently anaesthetized before receiving chronic bilateral implantation of guide cannulae (8.5 mm, 22 G, HRS scientific) targeting the PRh and EC (AP: -5.5 mm, ML: 5.5 mm, DV: -8 mm, at 10° angle relative to vertical axis). One rat died during surgery and 1 died during

behavioural testing due to complications associated with multiple implant replacements.

3.2.1.3 Hormone administration and assay measurement

The silastic tubing implanted at the nape of the neck contained 0.4 mg E2 (Sigma-Aldrich, St Louis, MO; E8875) in 8 mg cholesterol (Sigma-Aldrich; C8667). This technique has previously been shown to produce serum concentration levels of approximately 20 pg/ml for 21 days, consistent with naturally circulating levels of E2 during estrous phase of the rat estrous cycle (Mannino et al., 2005).

For Experiment 1A, rats were randomly assigned to either the High E group (n = 6) or the Low E group (n = 5). In addition to the chronic low E2 replacement provided by the implants, those in the High E group were also administered EB (10 µg in 0.1 ml sesame oil, s.c.; Sigma-Aldrich; E8515) 4 hr prior to the familiarization phase of each NOP trial. It has been shown that a 10 µg dose of EB administered systemically produces serum concentration levels that are comparable to the proestrous phase of the rat estrous cycle (Henderson et al., 1977). The Low E group received a vehicle control injection (0.1 ml sesame oil, s.c.). To mimic the hormonal conditions of the High E group, EB (10 µg in 0.1 ml sesame oil) was administered to a separate group of OVX rats not included in the behavioural procedure (N = 6, with chronic low E2 replacement via silastic implants) and blood was taken from the tail vein 4 hr later. Blood was also taken from the same rats 4 hr following oil administration to measure the hormonal conditions of the Low E group (chronic low E2 replacement + 0.1 ml

sesame oil). A 4-day delay occurred between the two periods of blood collection and the order in which they occurred was counterbalanced across rats. Immediately following each blood collection, samples were placed on ice, and centrifuged within 30 min. Plasma was collected and stored in -80°C until assayed. E2 levels were measured using commercially-available enzyme-linked immunosorbent assay (ELISA) kit (Immuno-Biological Laboratories Inc., Minneapolis, MI). The assay antibodies have 100% cross-reactivity with E2. The range of the assay is between 0 and 2000 pg/ml and the reported inter-assay variation is 7-9%.

For Experiment 1B, rats (*N* = 8) received bilateral infusions of E2 (β-cyclodextrin encapsulated E2, Sigma-Aldrich; E4389; 1.2 µl of 244.8 pg/µl on each side) dissolved in artificial cerebral spinal fluid (aCSF; 145.11 mM NaCl, 2.68 mM KCl, 2.13 mM $MgCl_2$, 1.96 mM Na_2HPO_4, 0.905 mM $CaCl_2*2H_2O$, 0.199 mM ascorbic acid) and vehicle (β-cyclodextrin, Sigma-Aldrich; C4767; dissolved in aCSF, 1.2 µl of 5.2 ng/µl on each side). The same OVX rats (with chronic low E2 via silastic implants) used in Experiment 1A to measure serum concentration levels of E2 following systemic injections of EB and oil were also used to measure peripheral E2 levels following intra-rhinal-cortical infusions mimicking the two condition in Experiment 1B. Blood was taken from the tail vein following the intracranial infusions of both E2 and vehicle and serum E2 levels were analyzed using an ELISA kit. A 4-day delay occurred between both collections. The order in which they occurred was also counterbalanced across rats.

3.2.1.4 Apparatus

An open field arena (60 cm X 70 cm X 70 cm) constructed of grey PVC plastic was used with stainless steel flooring covered with wood chip bedding. A video camera was positioned above the arena and both phases of the NOP test were recorded for later analysis.

Six objects for Experiment 1A and 12 for Experiment 1B served as test stimuli and were made of metal, glass, porcelain or glazed ceramic, varying 5-15 cm in height. There were three pairs of objects for Experiment 1A and 6 for Experiment 1B, with 3 copies of each object. Objects serving as novel stimuli were counterbalanced across rats in each group. Each pair had previously been screened to ensure that both objects evoked similar amounts of investigation in naive rats. The objects were affixed to the bottom of glass jars, and attached to the floor of the apparatus by screwing the jars into lids fixed in place. The objects were positioned 27 cm from opposing corners the arena.

3.2.1.5 Procedures

3.2.1.5.1 Experiment 1A. Eleven rats received two 5-min habituation sessions to the open field with 2 identical copies of an object, which was not used in any of the subsequent tests. Twenty-four hours later, rats received EB or oil administration followed 4 hr later by one 5-min *familiarization* session with two identical copies of an object (which served as the sample object). Following a retention interval of 72 hr, rats returned to the arena with a third copy of the sample object and a novel object for a 5-min test. A total of 3 NOP test trials were given to each rat spaced approximately one week apart. The same

hormone treatments were given prior to each trial. For an illustration of the experimental procedure, see *Figure 3.1*.

3.2.1.5.2 Experiment 1B. Nine rats first received two 5-min habituation sessions to the open field with 2 identical copies of an object. The familiarization phase of the first NOP trial occurred 24 hr later and was followed by infusions of either E2 or vehicle 10 min or 2 hr later. Rats returned to the apparatus 72 hr later for the 5-min retention test. A total of 4 NOP trials involved post-familiarization infusions and each rat received 1 trial under each infusion condition at each infusion delay (vehicle/no delay, vehicle/2-hr delay, E2/no delay, E2/2-hr delay), with the order in which each trial type was received was counterbalanced across rats. Two other trials involved infusions that occurred 10 min before familiarization (i.e. pre-familiarization infusion trials). Each rat received one trial in each infusion condition (vehicle and E2; *Figure 3.1*). Again, the order was counterbalanced across rats. A delay of approximately one week occurred between each NOP test trial.

3.2.1.6 Statistical Analyses

Results from all experiments are expressed as mean (+/- SEM) in *Figures* 3.2-3.4, and 3.7-3.9. All statistical analyses were conducted using SPSS version 20 for Windows (IBM, Chicago, IL) and type I error rate was set at $\alpha = .05$.

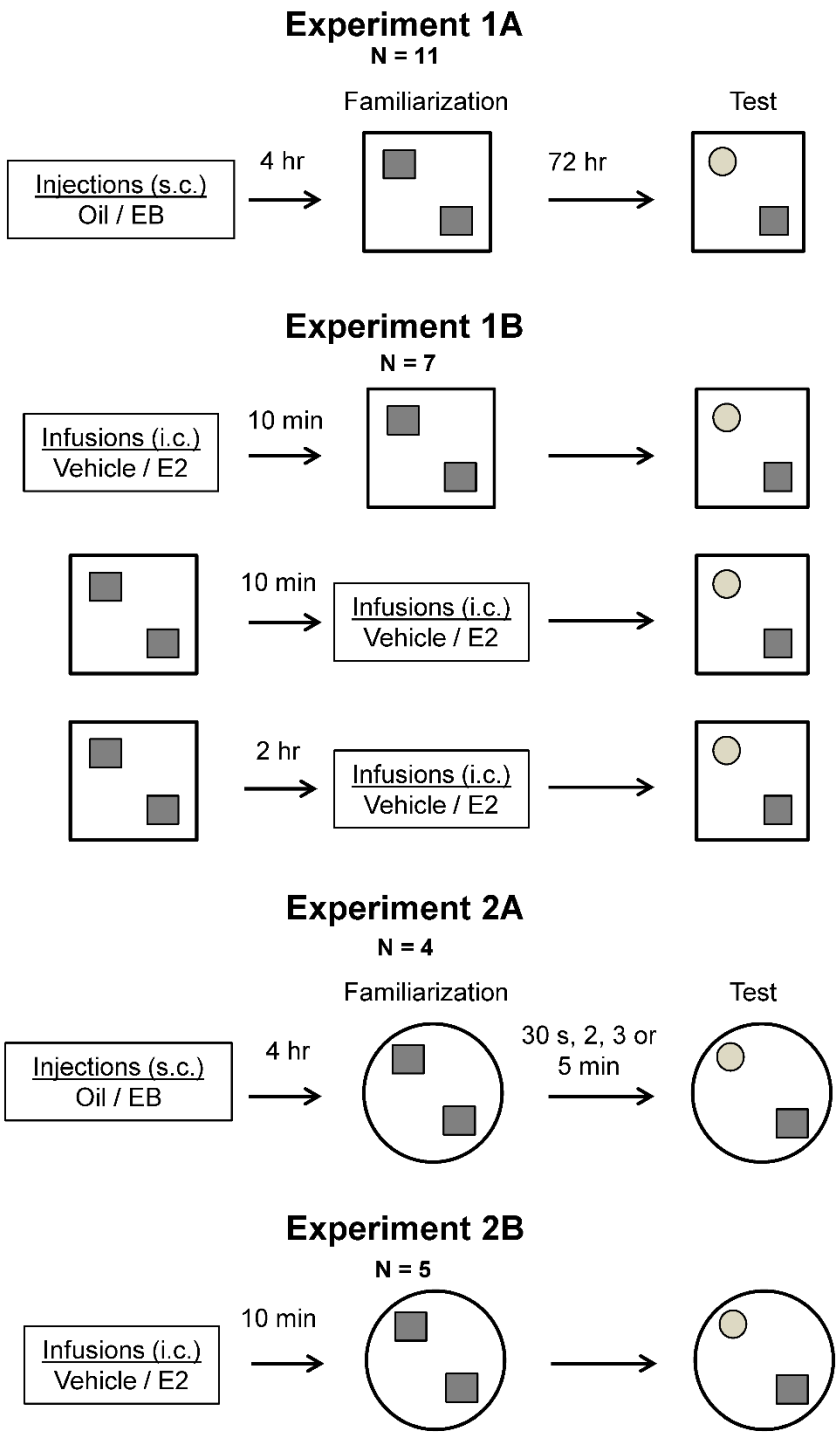

Figure 3.1: Experimental procedures for Experiments 1 and 2. Schematic diagram depicts procedure of both studies. Note: s.c. = subcutaneous injection; i.c. = intracranial infusion; EB = β-estradiol benzoate; E2 = 17-β estradiol.

Serum concentration levels of E2 following chronic low E2 replacement and high acute E replacement were compared using a paired-samples t-test (for Experiment 1A). A second paired-samples t-test was used to compare serum concentration of E2 levels following bilateral intra-rhinal-cortical infusions of vehicle and E2 infusions.

Time spent investigating each object during both familiarization and test phases was scored using ODLog version 2.7.2 (Macropod, software). An investigation ratio for the first 4 min of the test phase was calculated as the proportion of total time spent exploring the novel object (t_{novel}/ (t_{novel} + t_{sample})). This ratio served as the dependent measure for this test. A rat was considered to be investigating an object when rearing with at least one forepaw touching it, or when her head was within 3 cm of the stimulus. Biting, climbing, and sitting on the object was not considered object investigation.

For Experiment 1A, average investigation time during familiarization and retention tests for the 3 NOP test trials was obtained for each rat as were their average investigation ratios. Independent-samples t-tests were conducted comparing the high acute E replacement (High E) group to the chronic low E2 replacement (Low E) group on investigation time during familiarization and test, while 2 one-sample t-tests (one-tailed) were used to compare the mean investigation ratio of each group to chance performance (0.5). To determine whether object investigation during familiarization influences novelty preference, a Pearson correlation was conducted.

For pre-familiarization infusion trials in Experiment 1B, paired-samples t-tests were used to compare investigation time during familiarization and test for the two infusion conditions. Similar to Experiment 1A, investigation ratios obtained from the test phase were analyzed separately by condition using 2 one-sample t-tests (one-tailed). Investigation time during familiarization and test of the post-familiarization infusion trials were analyzed using 2X2 repeated-measures analysis of variance (ANOVA) with infusion condition and infusion delay as the 2 factors. Investigation ratios for the post-familiarization infusion trials were analyzed using 4 one-sample t-tests (one-tailed) and also with a 2X2 repeated-measures ANOVA. Average investigation time during familiarization and an average investigation ratio of each rat were calculated and used to obtain Pearson correlations for pre-familiarization and post-familiarization infusion trials.

3.2.2 Results

3.2.2.1 Experiment 1A

3.2.2.1.1 Assay Results. Serum concentration levels of E2 were statistically significantly higher in the High E group (*M* = 85.77 pg/ml, *SEM* = 24.11) than Low E group (*M* = 15.58 pg/ml, *SEM* = 1.38), t(5) = 2.974, p = .03, Hedge's g = 1.68.

3.2.2.1.2 NOP Test. During the retention test, the High E group demonstrated an above-chance preference, *t*(5) = 7.99, *p* = .000, Hedge's g = 0.86, whereas the Low E group did not, *t*(4) = 1.89, *p* = .07, Hedge's g = 0.37 (*Figure 3.*2B). The Low E group investigated objects for 45.15 s (*SEM* = 3.96), while the High E group investigated for 55.92 s (*SEM* = 6.32), which was not statistically different, *t*(9) = -1.37, *p* = .20, Hedge's g = .83 There was also no group difference during

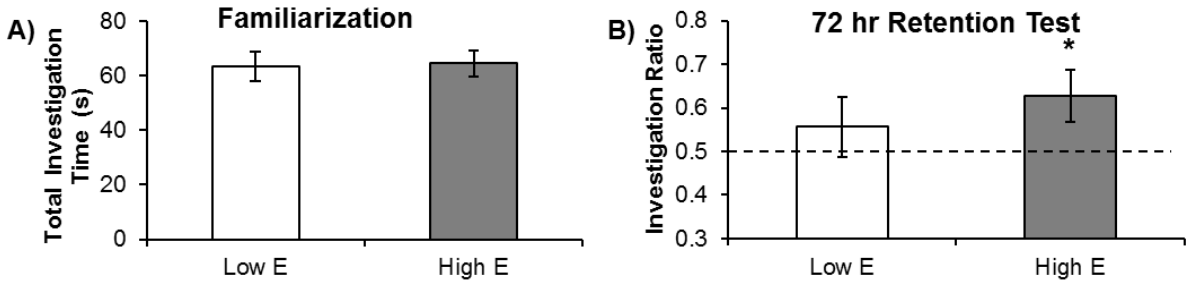

Figure 3.2: Object investigation for the NOP test in Experiment 1A following high acute E (High E) or chronic low E2 replacement (Low E). A. Mean time spent investigating the sample object during the 5-min familiarization phase. B. Investigation ratio obtained during the first 4 min of the 72-hr retention test. (* $p < .001$; one-sample *t*-test).

the familiarization phase, *t*(9) = 0.15, *p* = .89, Hedge's g = .09 as both groups spent approximately 65 s (Low E2: *M* = 63.45, *SEM* = 5.54; High E2: *M* = 64.51, *SEM* = 4.69) investigating the sample objects (*Figure 3.*2A).

3.2.2.2 Experiment 1B

3.2.2.2.1 Assay Results. Serum concentration levels of E2 were not different across the two intracranial infusion conditions (E2: *M* = 22.16 pg/ml, *SEM* = 0.99; Vehicle: *M* = 22.26 pg/ml, *SEM* = 1.87, t(5) = -0.08, p = .94, Hedge's g = 0.03.

3.2.2.2.2 NOP Test: Pre-familiarization Infusion Trials. Investigation ratios obtained during the retention tests were statistically significantly higher than chance-level after rats had received pre-familiarization infusion of E2, *t*(6) = 3.29, *p* = .01, Hedge's g = 1.24, but not vehicle, *t*(5) = -1.90, *p* = .06, Hedge's g = .78 (*Figure 3.3B*). Total object investigation during this phase (Vehicle: *M* = 55.25 s, *SEM* = 4.89; E2: *M* = 55.35 s, *SEM* = 5.66), *t*(5) = -0.04, *p* = .97, Hedge's g = .02, and during familiarization (Vehicle : *M* = 52.98 s, *SEM* = 7.14, E2: *M* = 45.89 s, *SEM* = 5.71; *Figure* 3.3A), *t*(6) = 0.82, *p* =.45, Hedge's g = 0.42, were not different across infusion conditions.

3.2.2.2.3 NOP Test: Post-familiarization Infusion Trials. Above-chance ratios (*Figure* 3.4B) during the retention test were obtained when E2 was infused immediately following the familiarization session, *t*(7) = 2.32, *p* = .03, Hedge's g = 0.82, but not 2 hr following familiarization, *t*(7) = 0.82, *p* =.22, Hedge's g = 0.29. Investigation ratios were no different from chance-level following vehicle infusions regardless of when they took place, *t*(7) = 0.32, *p* = .38, Hedge's g = 0.11 following no delay, *t*(7) = -0.95, *p* = .19, Hedge's g = 0.33 following a 2-hr

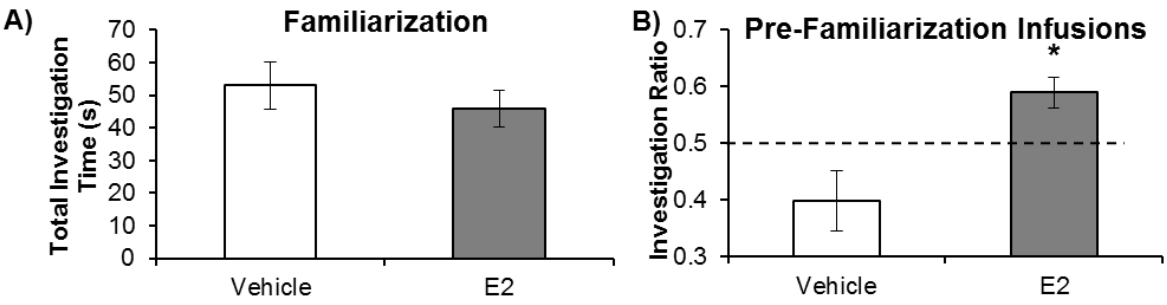

Figure 3.3: Object investigation during the NOP test in Experiment 1B following pre-familiarization infusions of E2 or vehicle. A. Mean time spent investigating the sample object during the 5-min familiarization phase. B. Investigation ratio obtained during the first 4 min of the 72-hr retention test. (* $p < .01$; one-sample *t*-test).

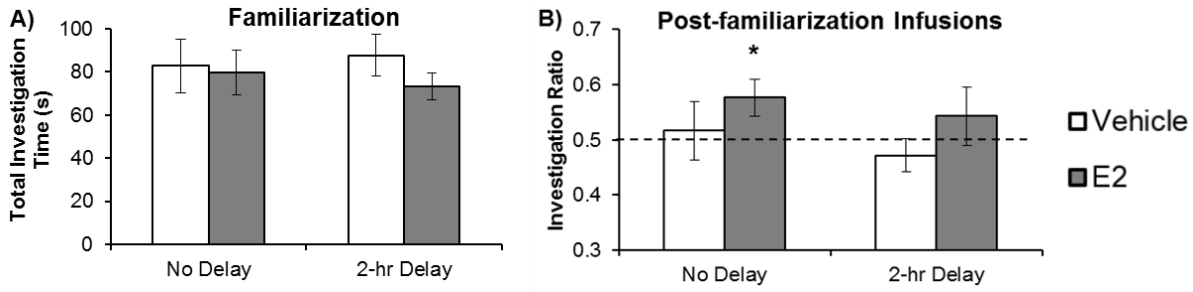

Figure 3.4: Object investigation during the NOP test in Experiment 1B for post-familiarization infusion of E2 or vehicle. A. Mean time spent investigating the sample object during the 5-min familiarization phase. B. Investigation ratios based on the first 4 min of the 72-hr retention test (* $p < .05$; one-sample *t*-test).

delay. A statistically significant main effect of infusion condition was found, $F(1, 7) = 9.66$, $p = .02$, partial $\eta^2 = .58$, with higher preference ratios following E2 infusion than vehicle. The infusion delay, $F(1, 7) = 0.99$, $p = .35$, partial $\eta^2 = .12$, and interaction, $F(1, 7) = 0.01$, $p = .92$, partial $\eta^2 = .002$, were not significant. Total investigation time during this phase revealed a statistically significant interaction between infusion type and infusion delay, $F(1, 7) = 8.83$, $p = .02$, partial $\eta^2 = 0.56$, but no main effects, Infusion condition: $F(1, 7) = 1.24$, $p = .30$, partial $\eta^2 = 0.15$, Infusion delay: $F(1, 7) = 0.46$, $p = .52$, partial $\eta^2 = 0.06$. Regardless of when E2 was infused, investigation time during the retention test did not differ across the 2 infusion delays (No delay: $M = 64.44$ s, $SEM = 5.32$; 2-hr delay: $M = 48.01$ s, $SEM = 4.32$), $t(7) = -2.69$, $p = .06$ (Bonferroni corrected), Hedge's g = 1.20. Similarly, no differences were observed between vehicle infusions that occurred immediately ($M = 56.96$ s, $SEM = 4.51$) and 2 hr after familiarization ($M = 65.54$ s, $SEM = 5.45$), $t(7) = -1.07$, $p = .64$ (Bonferroni corrected), Hedge's g = .6. The mean investigation time during familiarization for all post-familiarization infusion trials (*Figure* 3.4A) was 80.86 s ($SEM = 4.82$). No main effect of infusion condition, $F(1, 7) = 1.06$, $p = .34$, partial $\eta^2 = 0.13$, infusion delay, $F(1, 7) = 0.01$, $p = .92$, partial $\eta^2 = 0.002$, and no interaction, $F(1, 7) = 1.06$, $p = .34$, partial $\eta^2 = 0.13$, was found.

3.2.2.3 Correlations

For Experiment 1A, time spent investigating objects during familiarization was not statistically significantly correlated with the investigation ratio obtained during the test, $r = .04$, $p = .90$. There was also no statistically significant

correlation found in Experiment 1B, Pre-familiarization trials: r = .26, p = .62; Post-familiarization trials: r = -.41, p = .31.

3.3 Experiment 2

3.3.1 Material and Methods

3.3.1.1 Subjects

Ten female Long Evans rats (Charles River, Saint-Constant, Quebec) weighing between 175-200g at time of arrival served as subjects. These same rats were also used in Experiment 1B and were housed individually for both Experiment 2A and 2B.

3.3.1.2 Surgery

Following approximately two weeks of acclimation to the housing conditions, all subjects were ovariectomized bilaterally, silastic tube implants, and guide cannulae targeting the PRh and/or EC identical to that described in Experiment 1. In addition to the 2 deaths reported in Experiment 1B, a third rat died during behavioural testing for the DNMS task due to complications associated with multiple implant replacements. Thus, the final sample included 4 rats in Experiment 2A and 5 in Experiment 2B.

3.3.1.3 Hormone administration

Chronic low E2 replacement was provided to all rats in both Experiment 2A and B using the same silastic tube implants and the same amount of E2 and cholesterol as that described in Experiment 1. The same systemic injections (EB and oil) given in Experiment 1A were provided 4 hr prior to each DNMS test session for Experiment 2A. Unlike Experiment 1A, the protocol used in

Experiment 2A involved a within-subjects design; thus, DNMS task performance of each rat was obtained with both high acute E (chronic low E2 + acute 10 µg EB, s.c.) and chronic low E2 (chronic low E2 + oil, s.c.) replacement. Experiment 2B involved the same within design and the same intracranial infusions of E2 and vehicle as those described for the pre-familiarization infusion trials of Experiment 1B

3.3.1.4 Apparatus

A circular open field with grey PVC flooring (100 cm X 100 cm), and walls constructed from black fiberglass was used. Five food wells (~2.5 cm in diameter) were located at a distance approximately 22 cm from the wall and approximately 20 cm from each other. The array of the 5 wells made a circular shape on the floor. A metal cup (3 cm in diameter, 2 cm deep) containing approximately 100 food pellets, was fixed under each well. These pellets were inaccessible to the rats, but ensured that all wells smelled equally of food regardless of whether or not the well was baited (i.e., contained accessible food pellets). Familiarization and test sessions were video-recorded for subsequent behavioural analysis.

Approximately 150 objects with similar characteristics as those used in NOP test served as stimuli for the DNMS task. For objects serving as sample objects, 3 copies were used. Novel objects required one copy, however some objects that served as novel for some trials later served as sample objects and some objects serving as sample objects were also serving as novel objects during later trials. A minimal delay of two weeks was used between presentations

of an object. Each object was fixed to a glass jar, and inverted jar lids were attached to a small aluminum plate, approximately 4 cm x 10 cm. A small pin attached at one end of the aluminum plate extended downward 2.5 cm, and fit inside a small hole next to each food well, thus serving as a pivot around which the object could swivel. When an object was positioned over a food well, this mechanism enabled rats to easily displace it to gain access to the well beneath, without knocking over the object. A representation of the array of food wells and objects is presented in *Figure* 3.5.

3.3.1.5 Procedure

3.3.1.5.1 Experiment 2A. The procedure for the present DNMS task is adapted from previous ones (Aggleton, 1985; Kesner, 1991; Mumby & Pinel, 1994; Rothblat & Hayes, 1987). Each session consisted of several independent trials (4 in the present experiment), and training and testing occurred in four stages. Rats were initially shaped to displace objects to obtain food pellets from the well beneath (Dustless Precision Pellets, 45 mg, Bio-Serv, Frenchtown, NJ). Once a rat reliably displaced objects during both familiarization and test phase (across 2 consecutive trials), it continued to the next stage. The shaping required 15-22 trials, and 2 rats failed to reliably displace objects and so were excluded from the experiment. Next, the rats were trained using a brief retention interval of approximately 0.5 min between the familiarization and test phases of each trial; this *acquisition* stage continued for each rat until it consistently selected the novel object at a high level of accuracy (75% correct across 3 consecutive sessions). Eight rats were trained until they reached criterion performance, which required

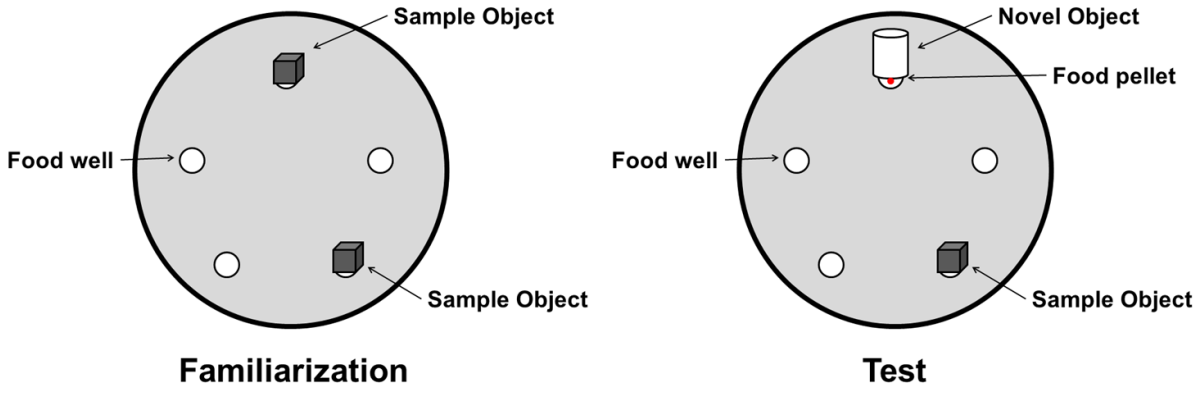

Figure 3.5: Schematic representation of the open field arena used for the delayed nonmatch-to-sample task. Diagram illustrates the array of food wells, placement of objects during both phases, and food reward located under the novel object during the test.

44-136 trials (*M* = 103.20, *SD* = 41.32). One rat failed to reach criterion performance within 136 trials and so was excluded. The remaining 7 rats were trained with progressively longer retention intervals. Each rat received 2 trials under each retention interval. Thus, at the end of this phase of training, all rats were following the procedural requirements of the DNMS task, and had experienced trials with each of the retention intervals that would be used in the ensuing stage of testing. DNMS task training occurred concurrent to Experiment 1B.

In the final stage, which is illustrated in *Figure* 3.*1*, rats (*N* = 4) received systemic injections (of 10 µg EB in 0.1 ml oil or 0.1 ml oil) 4 hr prior to each DNMS session. Performance was assessed across a range of retention intervals (0.5, 2, 3, and 5 min) on each session. Each rat (*N* = 4) received a total of 8 mixed-interval sessions. Four under chronic low E2 replacement (Low E, following administration of 0.1 ml sesame oil) and 4 under acute high E replacement (High E, following administration of 10 µg EB in 0.1 ml oil). Four mixed-interval sessions included trials with short retention intervals (2 trials with 0.5-min interval and 2 trials with 2-min interval per session), while the other 4 included trials with long retention intervals (2 trials with 3-min intervals and 2 trials with 5-min intervals per session). This resulted in 4 trials at each retention interval under each hormonal condition (Low and High E), for each rat. The order in which systemic injections and mixed-interval sessions were received was counterbalanced across rats. These trials were administered approximately 2 weeks after those administered for Experiment 2B (described below).

3.3.1.5.2 Experiment 2B. The same rats used in Experiment 2A were included in 2B with one additional rat, for a total of 5 subjects. DNMS testing was conducted as in Experiment 2A, but 10 min before the start of each DNMS session, rats received bilateral intracranial infusions of E2 or vehicle (*Figure* 3.1) and the order in which they occurred was counterbalanced across rats. Trials for Experiment 2B were administered several weeks after Experiment 1B was completed.

3.3.1.6 Statistical Analysis

Accuracy during training on the DNMS task used in Experiment 1B was calculated as percent of trials correct during each block of 4 trials. Performance during the first 4 training blocks (first 16 trials) was averaged together for each rat as was the final 4 training blocks (last 16 trials) and were both compared to chance (50%) using 2 one-sample t-tests (one-tailed). Similarly, performance on the mixed-interval sessions from Experiments 2A and 2B were calculated as percentage of trials correct at each retention interval (0.5, 2, 3, and 5 min) under each injection/infusion condition (oil/vehicle and EB/E2). Test performance from both Experiment 2A and 2B were analyzed using a 2X3 repeated-measures ANOVA with infusion condition and retention interval as within-condition factors. Planned comparisons (Bonferroni-corrected dependent-samples t-tests) were used to compare performance under each hormonal condition (Low and High E, for Experiment 2A) or intracranial infusion condition (vehicle and E2 for Experiment 2B) following each retention interval.

Latency (in seconds) to contact (with paw or nose) an object and displace it during the test phase of each test trial was scored using ODLog software

(Macropod). Latencies to contact and displace were averaged for trials at each retention interval and under each injection/infusion condition. Both sets of latency data for each experiment were analyzed using 2X3 repeated-measures ANOVA.

Performance on the DNMS task in Experiment 2B at 0.5, 2 and 3-min retention intervals was averaged separately for the E2-infusion condition. The averages of each rat (N = 5) were used to run a Pearson correlation to compare with investigation ratios obtain from NOP trials in Experiment 1B under the same infusion condition (i.e. E2). Specifically, investigation ratios from NOP trials involving pre-familiarization infusions of E2 were averaged with ratios obtained from trials involving post-familiarization infusions of E2 (no delay and 2-hr infusion delay). The same averages were obtained for performance on both tests for the vehicle condition. Thus, two Pearson correlations were calculated, one for the E2-infusion condition and the other for the vehicle-infusion condition.

3.3.2 Results

3.3.2.1 Histology

Following all behavioural procedures, rats from Experiments 1B and 2 received a lethal dose of sodium pentobarbital before transcardial perfusion took place with 0.9% saline (250 ml) followed by 10% formalin-buffered acetate (250 ml; Fisher Scientific). The brains were excised and stored in 10% formalin solution at 4°C until sectioning. Using a cryostat microtome, 40 µm coronal sections through the PRh and EC were sliced and mounted on gel-coated glass microscope slides. Cresyl violet staining was performed for histological confirmation of cannula placement.

Sections from 1 rat indicated incorrect cannulae placement and data from that subject were excluded from all analyses. Slices from two rats included in the sample indicated minimal unilateral damage to the ventral hippocampus resulting from guide cannulae. *Figure* 3.6A shows the bilateral location of cannula tips in the PRh and EC of each rat included in Experiments 1B and 2. A photomicrograph of a representative section is presented in *Figure* 3.6B and indicates the extent of damage that occurred due to cannula placement and multiple injections.

3.3.2.2 Experiment 2A

Results from the first 4 and final 4 training blocks are presented in *Figure* 3.7. Performance was not different from chance at the beginning of training (sessions 1-4; M = 47.5%, SEM = 13.95), $t(4) = -0.33$, $p = .38$, Hedge's g = 0.08 but was significantly better than chance by the end of training (sessions 31-34; M = 65.1%, SEM = 9.77), $t(2) = 7.00$, $p = .01$, Hedge's g = 0.81.

Performance under both circulating hormones conditions at each retention interval is presented in *Figure* 3.*8*. No main effect of hormone condition, $F(1, 2) = 2.53$, $p = .25$, partial $\eta^2 = .56$, retention interval, $F(2, 4) = 0.94$, $p = .46$, partial $\eta^2 = .32$, or interaction, $F(2, 4) = 3.59$, $p = .13$, partial $\eta^2 = .64$, was found for performance during the test trials. Rats performed better under low E than high E following a 0.5 min retention interval, $t(3) = 23.00$, $p = .000$ (Bonferroni corrected), Hedge's g = 1.96, but not following any other interval, $t(3) = 1.21$, $p = .96$ (Bonferroni corrected), Hedge's g = 0.93 (2-min interval), and $t(2) = 0.40$, $p = 2.19$ (Bonferroni corrected), Hedge's g = 0.33 (3-min interval).

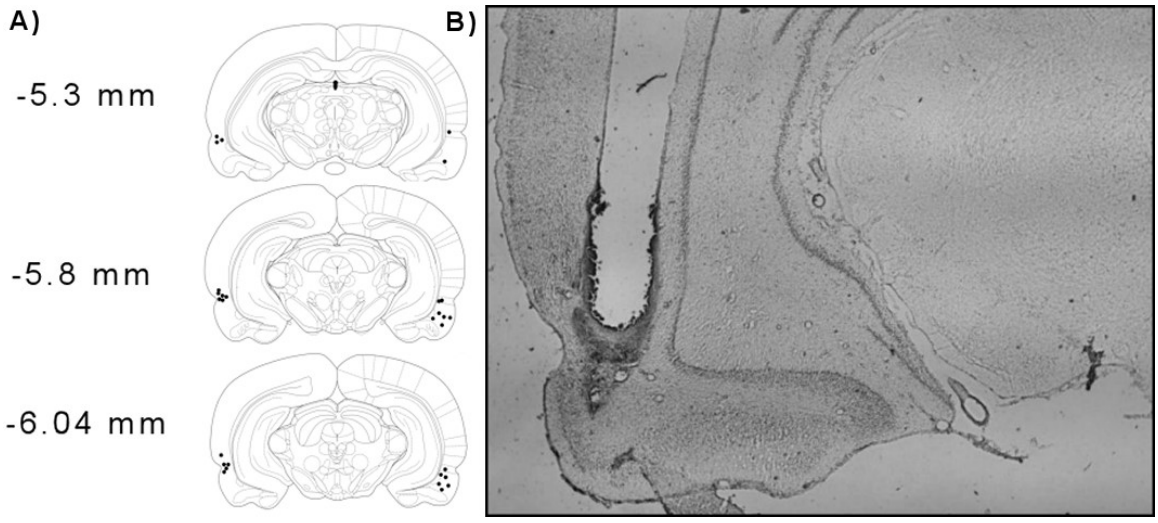

Figure 3.6: Representations of cannula placement for Experiments 1B and 2. A. Schematic representation of the placement of cannula tips (indicated with black circles) in both hemispheres of all rats included in Experiment 1B and 2 (*N* = 8). Some tips are presented in multiple sections. Values indicate distance from Bregma (Paxinos and Watson, 1998). B. Photomicrograph of stained tissue depicting placement of a guide cannula in the PRh with damage from injector extending into the dorsal portion of the EC.

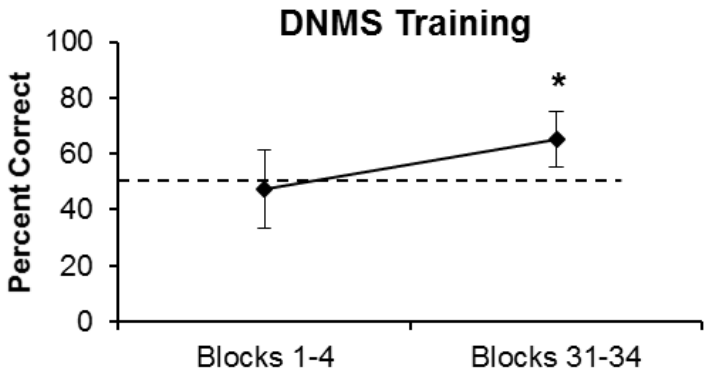

Figure 3.7: Percent trials correct during the training phase of the delayed nonmatch-to-sample task in Experiment 2. Training trials displayed in blocks of 4. The average percent correct for the first 4 blocks (first 16 trials) are at chance level (50%) and above chance for the final 4 blocks (last 16 trials; *$p < .05$; one-sample *t*-test).

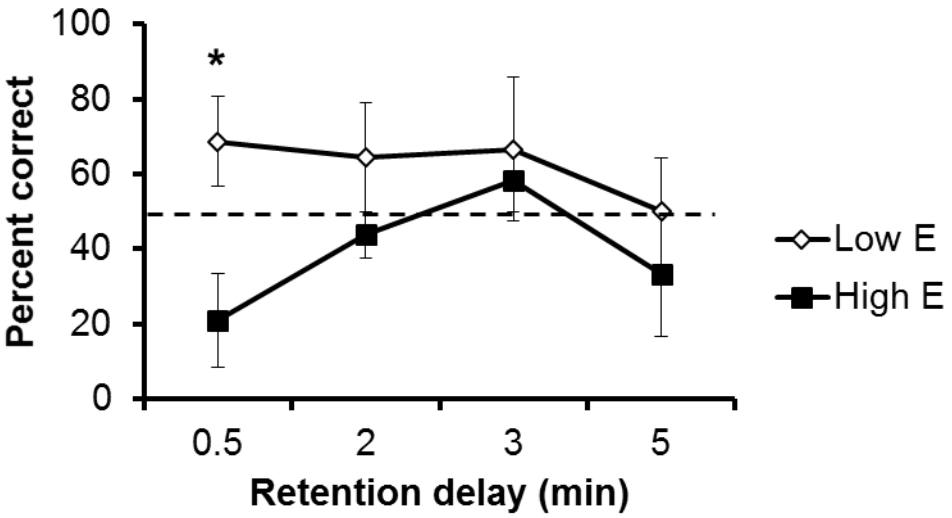

Figure 3.8: Performance on the delayed nonmatch-to-sample task of Experiment 2A. Percent trials correct during the test phase across 4 retention delays following systemic high acute E (High E) or chronic low E2 (Low E) replacement. (* $p < .001$; dependent-samples *t*-test).

Latency to contact an object during the test phase of the DNMS task was not influenced by hormone condition, $F(1, 1) = 0.49$, $p = .61$, partial $\eta^2 = .33$, retention interval, $F(3, 3) = 0.34$, $p = .80$ partial $\eta^2 = .25$, or the interaction between the two, $F(3, 3) = 0.96.$, $p = .51$, partial $\eta^2 = .49$. There was also no statistically significant main effect of hormone condition, $F(1, 1) = 0.64$, $p = .56$, partial $\eta^2 = .39$, retention interval, $F(3, 3) = 0.40$, $p = .77$ partial $\eta^2 = .29$, or interaction, $F(3, 3) = 1.16.$, $p = .45$, partial $\eta^2 = .54$, for latency to displace an object during the test phase.

3.3.2.3 Experiment 2B

Figure 3.9 shows the percent trials correct on the DNMS task at each retention interval following intracranial infusions of E2 or vehicle. A main effect of infusion condition, $F(1, 3) = 11.56$, $p = .04$, partial $\eta^2 = .79$ but no main effect of retention interval, $F(2, 6) = 0.25$, $p = .79$, partial $\eta^2 = .08$ or interaction, $F(2, 6) = 3.81$, $p = .085$, partial $\eta^2 = .56$ was found. Overall, rats under E2 performed worse than under vehicle. Performance was significantly worse following intracranial infusions of E2 than vehicle following a 3-min retention interval, $t(4) = 5.00$, $p = .045$ (Bonferroni corrected) Hedge's g = 1.40. No differences were found at any other retention interval, $t(4) = -0.54$, $p = 1.86$ (Bonferroni corrected), Hedge's g = 0.23 (0.5 min interval), and $t(3) = 1.58$, $p = .57$ (Bonferroni corrected), Hedge's g = 0.39 (2-min interval).

There were no differences found across infusion condition, $F(1, 3) = 4.43$, $p = .13$, partial $\eta^2 = .40$, retention interval, $F(3, 9) = 0.92$, $p = .47$, partial $\eta^2 = .24$, and no interaction between the two factors, $F(3, 9) = 0.60$, $p = .63$, partial η^2

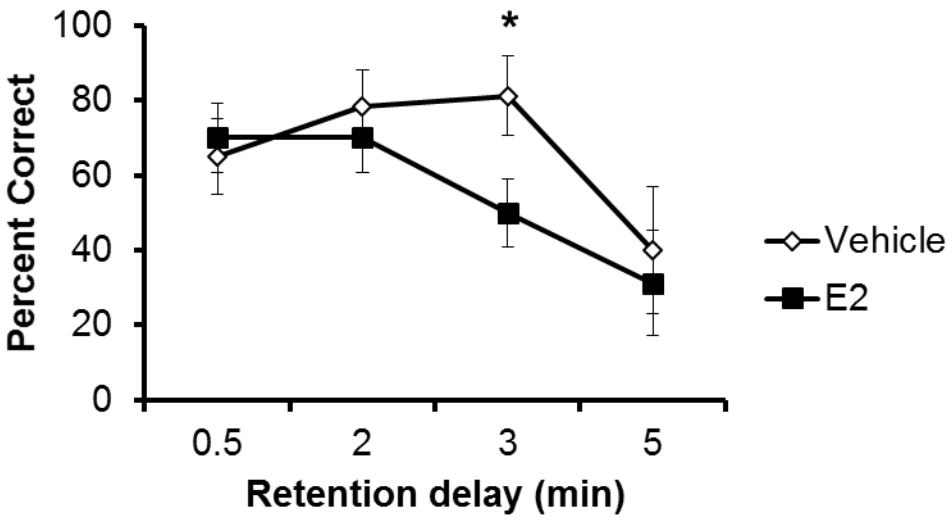

Figure 3.9: Performance on the delayed nonmatch-to-sample task of Experiment 2B. Percent trials correct during the test phase across 4 retention delays following vehicle and E2 infusions. (* $p < .05$; dependent-samples *t*-test).

= .17, for latency to contact an object. There was also no statistically significant main effect of infusion condition, $F(1, 3) = 0.28$, $p = .64$, partial $\eta^2 = .09$, or interaction, $F(3, 9) = 3.14$, $p = .08$, partial $\eta^2 = .51$, for latency to displace an object. However, there was a statistically significant effect of retention interval, $F(3, 9) = 7.35$, $p = .009$, partial $\eta^2 = .71$.

3.3.2.4 Correlations

Average investigation ratios obtained from NOP trials in Experiment 1B were not statistically significantly correlated with average performance on the DNMS task in Experiment 2B. This was found for trials following E2, $r = 0.16$, $p = .80$, and vehicle infusions, $r = -.29$, $p = .64$.

3.4 Discussion

OVX rats with systemic high acute E replacement displayed a significant novel-object preference following a 72-hr retention delay, whereas those with chronic low E2 replacement did not. These results are consistent with previous studies that found enhanced novelty preference following systemic administration of E2 (Fernandez & Frick, 2004; Gresack & Frick, 2006; Inagaki et al., 2010; Luine et al., 2003). Although the comparison group in the present study differed from previous ones in that chronic low E2 replacement was administered instead of no E2 replacement, the pattern of results are the same. When rats in the present study received infusions of E2 into the PRh/EC region, either before or immediately following exposure to the sample object, they later showed a significant novelty preference relative to rats that received vehicle infusion. There were no discernible effects on novelty preference when E2 was infused

intracerebrally following a post-familiarization delay of 2 hr. The latter result is consistent with a previous report of enhanced novelty preference following high acute E2 replacement or intra-HPC infusions of E2 given immediately, but not 3 hr following familiarization (Fernandez et al., 2008). The finding that high acute E levels affected novelty preference only if it occurred during or immediately after the familiarization phase, suggest that E modulates processes involved in consolidation. The results of Experiment 1B further suggest that E may affect novelty preference by modulating PRh and/or EC functions. While it is tempting to attribute the E2-mediated enhancement of novelty preference in Experiment 1 to an improvement in object-recognition memory, the data from the DNMS task in Experiment 2 suggest this interpretation is unlikely to be accurate. Rather than improving DNMS task performance -- as would be expected to occur if high acute E replacement resulted in improved ORM abilities -- our rats displayed significantly poorer performance on the DNMS task following either acute elevation of systemic E levels or infusion of E2 into the PRh/EC region. Thus, although the fact that high acute E altered NOP test performance only if it occurred during or immediately after the familiarization phase is consistent with the notion that E modulates processes involved in consolidation, this interpretation seems unlikely to be accurate, given the results on the DNMS task. Importantly, the hormone-treatment protocols were identical in Experiments 1A (NOP test) and 2A (DNMS task), and also in Experiments 1B (NOP test) and 2B (DNMS task), so the opposing effects of high systemic E levels or intra-PRh/EC infusions of E2 on NOP test and DNMS task performance cannot be easily

attributed to procedural variables related to factors other than the behavioural or cognitive operations which underlie normal performance on the NOP test and DNMS task.

In both Experiments 1 and 2, EB, an esterified form of E, was used to provide circulating E replacement. Use of this form of E results in a longer delay to clear it out of the blood stream and certain brain structures than the non-esterified form (β-estradiol).

One of the reasons we compared the effects of E on the NOP test and DNMS task is because concerns have been recently raised about the internal validity of the NOP test as a method of measuring object-recognition abilities in rodents. A major assumption of most investigators who employ the NOP test is that the magnitude of the novelty preference displayed on the test reflects the strength of the memory of the sample object. It is further presumed that rats encode information about the objects when they investigate them during the familiarization phase, and more encoding of object features should lead to stronger or more accurate memory for that object. We have not found a significant correlation between sample investigation time during the familiarization phase and preference ratios on the test, however, despite reviewing data sets from several experiments (Gaskin et al., 2010). Moreover, there was no significant correlation between these variables in the NOP performance of the present rats in either Experiment 1A or 1B. Correlational data are largely unreported in the literature, but personal communication between one of us (DGM) and other investigators who use the NOP test have confirmed that

the expected correlation repeatedly fails to show up. Importantly, experiments conducted to determine how sample-object investigation affects the magnitude of the novelty preference also failed to find the expected relationship. Gaskin et al. (2010) manipulated how much time rats were allowed to investigate the sample objects. Increasing investigation time from 5 to 30, or to 60 s, did not lead to a novelty preference on a test 3 hr later; further increasing investigation time to 90 s led to a significant novelty preference, but the magnitude of the preference was not affected by further increasing investigation time to 120 s. In another experiment, repeated exposure to the same sample objects, either within or across days, failed to alter the magnitude of the novelty preference (Gaskin et al., 2010).

The foregoing findings strongly suggest that the magnitude of the novelty preference is not a reliable indicator of the strength or accuracy of rats' memory of the sample object. It follows from this argument that the convention adopted by most investigators who use the NOP test – comparing the magnitude of the novelty preference under different treatments – is a dubious practice, if the goal is to gauge differences in ORM abilities. Notably, performance on the NOP test (average investigation ratio) was not correlated with performance on the DNMS task (percent correct across all trials). Comparing NOP test scores across treatment conditions may reveal differences in strength of a novel-object preference, but expressing a preference is not a feat of memory, and we therefore believe it is more sound to compare the ratios under each condition to what would be expected by chance, and from that analysis, decide simply

whether performance under a particular condition provides evidence that recognition occurred. Thus, we use a nominal scale (yes/no or yes/maybe) to compare performance across conditions with regards to object-recognition abilities.

The DNMS task can provide a relatively precise estimate of recognition abilities, as the percent-correct scores for each subject can be based on dozens or even hundreds of trials on which subjects make an explicit choice-response. There are good reasons to believe that a rat scoring 90% correct out of 100 DNMS trials has superior object-recognition abilities to a rat scoring only 65% (although alternative accounts for the different scores would need to be ruled out). It is less convincing to argue that a rat with a preference ratio of .90 on the NOP test has superior recognition abilities compared to a rat with a ratio of .65. In light of these considerations, we believe the results of the DNMS testing in Experiment 2 provide an accurate view of how high acute E levels, and intra-PRh/EC infusions of E2, affect ORM in OVX female rats. Accordingly, we doubt that the enhanced novelty preference in Experiment 1 was due to improved object-recognition memory. It is more likely that the effects of E on the NOP test reflect alterations in the *motivational* underpinnings of the rats' preference for novel objects.

Ovarian hormones have been suggested to influence the expression of preferences for settings associated with mating (Frye, Bayon, Pursnani, & Purdy, 1998), and these hormones appear to regulate conditioned place preference (Frye, 2007). These hormones also appear to modulate conditioned avoidance

responses (Diaz-Veliz, Burton, Benavides, Dussaubat, & Mora, 2000). Taken together, these data suggest that certain hormonal conditions, such as that seen in proestrous (when E2 is highest), may promote certain associations or preferences that are not formed under different hormonal conditions, such as in estrous (when E2 is lowest). This suggestion is consistent with studies demonstrating reinforcing properties of exploring novelty (Rebec, Christensen, Guerra, & Bardo, 1997). Increased exploration of a novel environment appears to occur in female rats when E2 levels are highest (Archer, 1975). Thus, it is plausible that high acute E replacement and intra-PRh/EC infusions of E2 enhanced novelty preference during the retention test as the reinforcing properties of novel-object exploration occur under this hormonal condition, while being absent when E2 replacement is low. Although novelty preference was observed only when E administration occurred before or soon after familiarization, it did not occur when administered following a 2-hr delay. For E administration to promote novelty preference via this proposed association, it would need to occur in close proximity to familiarization, so it is entirely possible that a delay of 2 hr would be too long for an association to be made.

It is important to consider that the E2-mediated disruption of DNMS task performance when infused into the PRh/EC region might not reflect an object-recognition impairment. Any memory test can be failed for reasons other than failure of memory, such as changes in motivation, attention, or an impaired ability to remember procedural aspects of task performance. Several incidental observations seem to rule out these factors as the basis for the DNMS

impairments observed in Experiment 2B. All rats were well-trained on the DNMS task before the experimental treatments were administered, and a within-subjects design was used both in Experiment 2A and 2B. No obvious changes in behaviour were evident in the video-records of rats performing the DNMS task under different treatment conditions. For example, rats' latencies to approach and displace one of the objects during DNMS test phases were not different following E or control treatments, and this was the case in both the systemic-administration experiment (2A), and the intra-PRh/EC infusion experiment (2B). Rats readily retrieved food pellets from the wells and immediately consumed them, and this did not vary across treatment conditions in either experiment. The only significant differences in DNMS performance across treatment conditions were in the accuracy of the animals' choices, which was worse when rats had high systemic levels of E than when E levels were lower, and also worse when E2 was infused into the PRh/EC region than when the vehicle alone was infused.

It is important to note that the longest retention intervals we used in the DNMS task were only a few minutes long, and this considerably shorter than the 72-hr retention interval we used with the NOP test. These differences were unavoidable for two reasons: 1) Rats cannot achieve reliably accurate scores on the DNMS task with longer retention intervals. 2) A relatively long interval of a 72 hr was used on the NOP test to ensure that rats did not display a significant novelty-preference under control conditions, which would have possibly obscured any E-mediated enhancement. Nonetheless, we must consider that one possible reason for the discrepant effects of E on DNMS task and NOP test performance

might be that this hormone has a generally detrimental effect on short-term memory, while enhancing long-term memory. It is not clear what set of known mechanisms could have such opposing consequences for long-term and short-term memory, so we favor the view that the effects of E on the NOP test are not due to enhanced long-term object-recognition memory. Unfortunately, we do not currently have the behavioural tools that would be needed to directly test that hypothesis. The discrepant results may also be due to elevated levels of E in serum or specifically in PRh/EC during the test phase of the DNMS task while being absent during the retention test of the NOP test. Future studies can address this issue by observing whether the presence of high E during the test phase of the NOP test eliminates the novel-object preference observed in the present study.

The intracranial infusions of E2 were given at a concentration that is approximately twice as high as concentrations used previously (Packard & Teather, 1997b; Zurkovsky et al., 2007). Since some of the β-cyclodextrin in the compound may not contain E2, the approximate E2 concentration is likely an overestimation. Thus, to ensure high levels of E2 in the regions of interest, a higher concentration was chosen.

The fact that our E2 infusions targeted both the PRh and EC makes it unclear as to whether E2 acts in only one or both regions to produce the observed results. Moreover, the effects on NOP performance of E2 infusions into PRh/EC resemble those that have been previously reported following E2 infusions into the rodent HPC. Mice displayed a significant novel-object

preference after E2 (5 μg) was infused into the HPC immediately following the familiarization phase, but not after vehicle infusion, or after E2 infusion that was delayed until 3 hr following familiarization (Fernandez et al., 2008). It may be the case that similar neurobiological mechanisms underlie the E2-meidated enhancement of novelty-preference when it is infused either into the HPC, the PRh, or the EC. Our data cannot begin to address that possibility, but they do place boundaries on what should be viewed as adequate functional interpretations of certain behavioural changes that result from high acute E levels or elevated E2 in the HPC or in parahippocampal cortical areas. The DNMS task results suggest that our rats' object-recognition abilities were not enhanced by infusion of E2 into the PRh/EC region, and in fact they may have been impaired, so it is likely the enhanced novelty preference was due to something other than improved object-recognition abilities. One reason to suspect the same thing about previous findings of enhanced novel-object preference following E2 infusion into the HPC (Fernandez et al., 2008) is that rats with extensive HPC lesions display a significant novelty-preference on the NOP test (Forwood, Winters, & Bussey, 2005; Gaskin, Tremblay, & Mumby, 2003; Gaskin et al., 2010; Good, Barnes, Staal, McGregor, & Honey, 2007; Langston & Wood, 2010; Mumby, Tremblay, Lecluse, & Lehmann, 2005; Winters, Forwood, Cowell, Saksida, & Bussey, 2004); therefore, the ability to demonstrate object recognition on the NOP test does not depend critically on HPC functions. Furthermore, performance on DNMS tasks is not significantly affected by HPC damage (Mumby, Wood, & Pinel 1992; Mumby, 2001), whereas DNMS performance in

rats is significantly impaired by PRh lesions (Mumby & Pinel, 1994; Wiig & Bilkey, 1995). The effects of systemic high acute E or intra-PRh/EC infusion of E2 thus mimic the effects of PRh lesions on the DNMS task, which is consistent with our conclusion that these hormone treatments had detrimental effects on the rats' object-recognition abilities.

One final point to consider is that the present experiments utilized a control condition under which circulating levels of E2 were comparable to those that naturally occur in the estrous phase. Previous studies compared OVX rodents with high acute E replacement to those with no replacement (Fernandez & Frick, 2004; Gresack & Frick, 2006; Inagaki et al., 2010; Luine et al., 2003), and the latter condition may not be an ideal control as it does not correspond to anything that occurs in natural-cycling females. OVX produces circulating levels of E2 representative of what is experienced during post-estropause, and so are much lower than what is experienced in cycling rodents. Since OVX removes variability in circulating E2 levels, it is an ideal procedure to use when investigating the effect of specific E2 levels on cognitive abilities. Rather than removing most of the circulating E2 levels, physiologically-relevant E2 levels can be maintained to provide a control group with chronic low E2 replacement.

CHAPTER 4

Intra-perirhinal estrogen receptor beta and object-recognition memory in ovariectomized rats

Preface

The study described in Chapter 3 found contrasting effects of intra-PRh/EC on the NOP test and DNMS task. The findings were taken to suggest that E2 impairs ORM via actions within these structures. There is some evidence suggesting that E2 influences novelty preference via ERβ. The present chapter sought to determine whether intra-PRh infusion of a selective ERβ agonist impairs ORM. OVX females with chronic low E2 replacement (~20 pg/ml, serum) received bilateral intra-PRh infusion of vehicle, E2 (244.8 pg/µl), or DPN (2 µg/µl), then were tested on either the NOP test, or DNMS task. The general findings obtained in Chapter 3 were replicated in the present study. Novelty preference was observed following intra-PRh infusions of E2 at a 4-hr retention delay, whereas no preference was observed following vehicle infusions. Intra-PRh infusions of E2 resulted in lower accuracy on the DNMS task at a 5-min retention delay relative to vehicle infusions. E2 appears to enhance novelty preference via actions at ERβ in the PRh, as DPN infusions resulted in novelty preference following a 4- and 72-hr retention delay. However, DPN did not influence performance on the DNMS task. While E2 appears to enhance novelty preference following both a 4- and 72-hr retention delay via ERβ, E2 likely impairs DNMS task performance via another estrogen receptor in the PRh.

Abstract

17-β estradiol (E2) enhances novel-object investigation when administered around the time rodents are first presented with a familiar object. There is some evidence suggesting E2 enhances novelty preference via estrogen receptor (ER) β. Given the role of the perirhinal cortex (PRh) in object-recognition memory, the aim of the present study was to determine whether E2 binds to ERβ in this structure to influence performance on the NOP test and the delayed nonmatch-to-sample (DNMS) task. Ovariectomized (OVX) rats (*n* = 10) received chronic low E2 (~20 pg/ml serum) replacement, then intra-PRh infusion of E2 (244.8 pg/ml), diarylpropionitrile, a selective ERβ agonist (DPN, 2 μg/μl), or vehicle before each NOP test trial, and were tested either 4 or 72 hr later. A different set of OVX rats (*n* = 7) received the same infusions before the DNMS task, using 4 retention delays (0.5 – 5 min). Intra-PRh DPN enhanced novelty preference following both retention delays, but had no effect on DNMS task performance. Consistent with previous research, intra-PRh E2 enhanced novelty preference (following a 4-hr retention delay only), and impaired DNMS task performance following a 5-min retention delay. The results from the NOP test suggest that when E2 binds to ERβ in the PRh, it influences non-cognitive processes that contribute to the expression of novelty preference. This is consistent with the results of the DNMS task, as DPN had no effect on performance. While E2 appears to enhance novelty preference via intra-PRh ERβ, it may influence object-recognition memory via another estrogen receptor.

4.1 Introduction

Enhanced object-recognition memory (ORM), which is the ability to discriminate the familiarity of objects previously encountered (Aggleton & Brown, 1999), has been observed in humans and non-human primates following elevated levels of 17-β estradiol (E2). Surgical or pharmacologically-induced ovariectomy (OVX) in humans or non-human primates is associated with reduced accuracy scores on either the delayed nonmatching-to-sample (DNMS) or match-to-sample (DMS) tasks relative to and estrogen (E) replacement condition (Craig et al., 2010; Lacreuse, Herndon, & Moss, 2000; Voytko, Higgs, & Murray, 2008). However, one study reported reports no effect of E2 replacement on DNMS task performance (e.g. Lacreuse & Herndon, 2003).

Using a rodent version of the DNMS task, OVX rats with chronic low E (~20 pg/ml serum via silastic implants) demonstrated superior performance on the DNMS task relative to those with chronic low E plus acute high E replacement (10 μg estradiol benzoate, s.c; Gervais et al., 2013). These results support the idea that higher E levels are associated with impaired, not improved ORM. The discrepant pattern between this study and those described above might reflect differences in the comparison condition. The studies conducted in humans and non-human primates compared naturally cycling or E replacement (chronic low E2, 40-80 pg/ml, plus acute high E2 0.10-0.20 mg/ml every 12 days) to an OVX (i.e. non-cyclic, very low E2 condition) group. In our study (Gervais et al., 2013), the comparison condition did not involve an OVX group without E replacement, but rather a chronic low E replacement condition. Although E

replacement may result in superior performance on the DNMS/DMS task compared to a no replacement condition, there may be a dose-dependent effect of E on this ability, whereby lower levels produce improved performance relative to higher levels. In addition to dose, other factors that can influence whether E enhances or impairs learning and memory includes the ability being assessed, hormone regimen, interaction with other hormones, mode of delivery, sex, stress and motivation (Korol, 2004; Zurkovsky, Brown, Boyd, Fell, & Korol, 2007).

ORM is dependent upon the functioning of the perirhinal cortex (PRh; for a review, see Winters, Saksida, & Bussey, 2008). One recent study reported impaired DNMS task performance at a 3-min retention delay following intra-rhinal cortical (PRh and EC) infusions of E2 (244.8 pg/µl) in OVX rats given chronic low E2 replacement (~20 pg/ml serum; Gervais et al., 2013). All three estrogen receptors (ERα, ERβ, GPER1) are expressed in this region (Blurton-Jones & Tuszynski, 2002; Brailoiu et al., 2007; Hazell et al., 2009; Shughrue, Lane, & Merchenthaler, 1997; Shughrue & Merchenthaler, 2001), and there is evidence that ERβ is more highly expressed in the PRh (Shughrue et al., 1997). Therefore, the purpose of the present study was to determine whether E2 binds to ERβ in this structure to influence ORM in OVX rats with chronic low E2 replacement.

In addition to the DNMS/DMS tasks, investigations into the modulatory effect of E2 on ORM in rodents use the Novel-Object Preference (NOP) test. Studies report impaired performance on the NOP test (i.e. novelty preference) following OVX (Fernandez & Frick, 2004; Gresack & Frick, 2006; Inagaki, Gautreaux, & Luine, 2010 Luine, Jacome, & MacLusky, 2003; Phan et al 2012).

or chronic low E2 replacement (~20 pg/ml serum via silastic implants; Gervais et al., 2013) relative to moderate to high E replacement (rats: 5-30μg/kg acute; mice: 1.5 μg/kg -0.4 mg/kg, acute, s.c.; 1-2.5 μM, chronic, p.o.). Intra-PRh infusions of E2 (244.8 pg/μl) also enhance novelty preference on a 72-hr retention test (Gervais et al., 2013). Unlike the results from the DNMS task used by Gervais and colleagues (2013), studies using the NOP test support the idea that E2 acts within the PRh to enhance ORM in rodents following delays ranging from 5 min to 72 hr.

While intra-PRh infusions of E2 enhanced novelty reference on a 72-hr retention test, they impaired DNMS task performance in the same set of rats. The effect of E2 on accuracy on the DNMS task was delay-dependent, as performance was influenced following a 3-min retention delay, but not following briefer (0.5-2 min) delays. Incorporating NOP test trials with different retention delays can help clarify the divergent effect observed on both tests. Any effect on novelty preference following a long (i.e. 72 hr), but not shorter (i.e. 4 hr) retention delay is consistent with an effect on long-term ORM.

Vaucher and colleagues (2002) investigated whether novelty preference is influenced by chronic E2 replacement (~12-18 ng/ml serum via silastic implants) in OVX mice following retention delays of 3 hr and 6 hr (Vaucher et al., 2002). Novelty preference was higher following E2 replacement relative to a no replacement group, and this difference was not delay-dependent. Although the authors concluded that the enhanced novelty preference reflects improved ORM ability, two important limitations need to be considered. The first concerns group

comparisons that were made. A previous study demonstrated that differences in in the magnitude of preference ratios do not reflect differences in the strength of ORM (Gaskin et al., 2010). Since the magnitude of the preference ratios were compared by Vaucher and colleagues (2002) without determining whether novelty preference was observed in both groups, it is possible that both groups demonstrated novelty preference. If this is indeed what occurred, the difference in the magnitude of the preference is uninterpretable, and so E2 replacement may not have promoted novelty preference in this particular study.

The second limitation to the study by Vaucher and colleagues (2006) concerns the retention delays used. The retention delays used were 3 hr apart and so may not have been sufficiently different for delay-dependent effects to be observed. A more recent study tested OVX mice with and without E2 replacement on the NOP test following a 24-hr and 48-hr retention delay (Gresack & Frick, 2006). Novelty preference was observed following both retention delays in the E2 replacement (0.2 & 0.4 mg/kg, i.p.) conditions, but not in the no E2 replacement condition. Since both studies failed to report delay-dependent effects, it is possible that the chosen retention delays within each study were not sufficiently different to produce such an observable effect. Ideally, the shortest retention delay should be brief enough to result in reliable demonstration of novelty preference, and the longest delay resulting in novelty preference barely above chance level. Therefore, perhaps combining the retention delays used by both studies may result in a delay-dependent effect. Additional studies using NOP test trials with a wider range of retention delays is

necessary before conclusions can be made about whether enhanced novelty preference reflects improvements in long-term ORM. For example, intra-E2 infusions enhancing novelty preference following a 72-hr, but not 4-hr retention delay on the NOP test is consistent with an enhancement of long-term ORM.

There is some evidence that E2 influences novelty preference by binding to ERβ (Jacome et al., 2010; Walf, Koonce, & Frye, 2008; Walf, Koonce, Manley, & Frye, 2009). For example, acute administration of diarylpropionitrile (DPN, 3 mg/kg, s.c.), a selective ERβ agonist, to OVX rats results in novelty preference whereas administration of propyl pyrazole triol (PPT, 3-5 mg/kg, s.c.), a selective ERα agonist, or vehicle does not. The novelty preference observed in the DPN-treatment condition occurred when administered before and immediately following familiarization (Jacome et al., 2010), which is consistent with the idea that E2 (via ERβ) enhances processes involved in the consolidation of object representations. However, one study reported an effect of a selective of ERα, but not ERβ, in novelty preference in OVX mice (Phan, Lancaster Armstrong, MacLusky, & Choleris, 2011), and another that reported no effect of either ligand on DNMS task performance in macaques (Lacreuse, Wilson, & Herndon, 2009). Should E2 influence ORM by binding to ERβ, perhaps this occurs in the PRh.

The present study investigated whether E2 modulates ORM via ERβ within the PRh. OVX rats under chronic low E2 replacement (~20 pg/ml serum) received intra-PRh infusions of E2, DPN, and vehicle prior to the NOP test or DNMS task. To determine whether E2 has delay-dependent effects on novelty preference (consistent with a role in ORM), a 4- and 72-hr retention delay was

used for the NOP test. DNMS task performance following four retention delays (0.5, 2, 3, and 5 min) was also compared across the same three infusion conditions.

4.2 Materials and methods

4.2.1 Subjects

Thirty-six Long Evans female rats (bred in house) weighing between 240 and 440 g at the time of surgery served as subjects. Rats were housed in pairs in transparent shoebox cages lined with a combination of woodchip and corncob bedding under 12:12 reverse light cycle (lights on at 8:00 pm) with ad libitum access to water and ~25 g daily access to chow. Following surgery, rats were housed individually for the remainder of the study. Approval for all procedures was provided by Concordia University's Animal Research Ethics Committee in accordance with the guidelines established by the Canadian Council on Animal Care.

4.2.2 Surgery

Surgeries occurred prior to behavioural testing for the NOP test (n = 12) or following successful DNMS task training (n = 16). Rats were anesthetized with isoflurane (Jaansen, Toronto, Ontario, Canada) before receiving chronic bilateral implantation of guide cannulae (8.5 mm, 22 G, HRS Scientific) targeting the PRh (AP: -5.5 mm, ML: +/- 6.0 mm, DV: - 7 mm at 10° angle relative to vertical axis). Bilateral ovariectomy using a standard aseptic procedure through a lumbar incision was also performed. In addition, 1 cm silastic tubing (i.d. 1.47 mm, o.d 1.96 mm) containing E2 in cholesterol was implanted at the nape of the neck.

The implants were replaced every four weeks (2 times total), and a delay of 7 days occurred between implantation and data collection. This ensured stable serum E2 levels throughout the 21-day period during which time behavioural testing occurred. Four rats did not survive surgery and three died due to complications associated with multiple implant replacements.

4.2.3 Hormone administration

The silastic implants contained 0.4 mg E2 (Sigma-Aldrich, St Louis, MO) and 8 mg cholesterol (Sigma-Aldrich), which has been shown to produce serum concentration levels comparable to the estrous phase (Gervais et al., 2013; Mannino et al., 2005). Immediately prior to each trial, rats received bilateral infusions of dialrylpropionitrile (DPN; 2 μg/μl, 1 μl/side) dissolved in 0.9% saline with 25% β-cyclodextrin, E2 (water-soluble E2, Sigma-Aldrich; 244.8 pg/μl, 1 μl/side) dissolved in 0.9% saline, or vehicle (1 μl/side).

4.2.4 Apparatuses

4.2.4.1 NOP test. An open field arena (60 cm X 70 cm X 70 cm) constructed of grey PVC plastic was used. The flooring was made of stainless steel and was covered with woodchip bedding. A video camera was positioned above the arena and all trials were recorded for behavioral analysis.

A total of 24 objects (one pair per trial) served as test stimuli and were made of porcelain, ceramic, metal, glass, or plastic, and varied 5-15 cm, in height. Three copies of each object were used, and objects serving as novel stimuli were counterbalanced across rats in each condition. Each pair had been previously been screened to ensure that both objects evoked similar amounts of

investigation in naïve rats. Objects were affixed to the bottom of glass jars, and attached to the floor of the apparatus by screwing the jars into lids fixed in place. The objects were positioned 27 cm from opposing corners of the arena.

4.2.4.2 DNMS task. A circular open field with grey PVC flooring (100 cm X 100 cm), and walls constructed from black fiberglass was used. Five food wells (~2.5 cm in diameter) were located at a distance approximately 22 cm from the wall and approximately 20 cm from each other. The array of the 5 wells made a circular shape on the floor. A metal cup (3 cm in diameter, 2 cm deep) containing approximately 100 food pellets, was fixed under each well. These pellets were inaccessible to the rats, but ensured that all wells smelled of food regardless of whether or not the well was baited (i.e., contained accessible food pellets). Familiarization and test sessions were video-recorded for subsequent behavioural analysis.

Approximately 150 objects with similar characteristics as those used in NOP test served as stimuli for the DNMS task (Gervais et al., 2013). For objects serving as sample objects, 3 copies were used. Novel objects required one copy, however some objects that served as novel for some trials later served as sample objects and some objects serving as sample objects were also serving as novel objects during later trials. A minimal delay of two weeks was used between presentations of an object. Each object was fixed to a glass jar, and inverted jar lids were attached to a small aluminum plate, approximately 4 cm x 10 cm. A small pin attached at one end of the aluminum plate extended downward 2.5 cm, and fit inside a small hole next to each food well, thus serving as a pivot around

which the object could swivel. When an object was positioned over a food well, this mechanism enabled rats to easily displace it to gain access to the well beneath, without knocking over the object.

4.2.5 Procedures

4.2.5.1 NOP test. Rats received two 5-min habituation sessions to the open field with 2 identical copies of an object. Twenty-four hours later, rats received infusions of vehicle, E2 or DPN and 10 min later, were re-introduced to the open field with 2 copies of another object (i.e. sample objects) for the 5-min familiarization phase. Either 4 hr or 72 hr later, rats returned to the apparatus for the 5-min retention test, whereby a third copy of the sample object and a novel object were presented. A total of 6 NOP test trials were given to each rat spaced approximately 2 days apart. Each rat received one trial under each infusion condition (vehicle, E2, and DPN) for each retention delay (4-hr and 72-hr). The order for each infusion condition was counterbalanced across rats.

4.2.5.2. DNMS task. The procedure is presented elsewhere in detail (Gervais et al., 2013). Each session consisted of several independent trials (4 in the present experiment), and training and testing occurred in four stages: Rats were initially shaped to displace objects to obtain food pellets from the well beneath (Dustless Precision Pellets, 45 mg, Bio-Serv, Frenchtown, NJ). Once a rat reliably displaced objects during both familiarization and test phase (across 2 consecutive trials), it continued to the next stage. The shaping required 8-58 (M = 28.89, SEM = 4.61) trials, and 5 rats failed to reliably displace objects and so were excluded from the experiment. Next, the rats were trained using a brief

retention delay of approximately 0.5 min between the familiarization and test phases of each trial; this *acquisition* stage continued for each rat until it consistently selected the novel object at a high level of accuracy (75% correct across 5 consecutive sessions). Sixteen rats were trained until they reached criterion performance, which required 24-148 trials (M = 78.25, SEM = 9.97). The remaining 3 rats did not reach criterion performance after 160 training trials and so were thus removed from the experiment. Following training, rats underwent surgery. Three rats did not survive surgery and another 2 rats experienced complications as result of surgery and were euthanized. The remaining 11 rats were trained with progressively longer retention delays. Each rat received 4 trials under each delay. Thus, at the end of this phase of training, all rats were following the procedural requirements of the DNMS task, and had experienced trials with each of the retention delays that would be used in the ensuing stage of testing.

In the final stage, rats received the intra-cranial infusions prior to each DNMS session. Performance was assessed across a range of retention delays (0.5, 2, 3, and 5 min) on each session. Each rat received a total of 12 mixed-interval sessions (4 under each infusion condition). Four mixed-interval sessions included trials with short delays (two trials with 30-s interval and two trials with 2-min interval per session), while the other 4 included trials with long delays (two trials with 3-min intervals and two trials with 5-min intervals per session). This resulted in 4 trials at each retention delay under each hormonal condition, for each rat.

4.2.6 Statistical Analysis

Results are expressed as mean (+/- SEM) in *Figures 4.4-4.6*. All statistical analyses were conducted using *Statistical Program for the Social Sciences* software (IBM, Chicago, IL) and type I error rate was set at $\alpha = .05$.

4.2.6.1 NOP test. Time spent investigating each object during familiarization and test phases was scored using ODLog (Macropod, software). A rat was considered to be investigating an object when rearing with at least one forepaw touching it, or when its head was within 3 cm of the stimulus. Biting, climbing, and sitting on the object were not considered object investigation. An investigation ratio for the first 4 min of the test phase for each trial was calculated as the proportion of total investigation time spent exploring the novel object ($time_{novel}/(time_{novel} + time_{familiar}$). These ratios were averaged separately by condition under each retention delay. One-sample t-tests were used to compare ratios obtained under each infusion condition and following each retention delay to chance (0.5) level. In addition, two factorial within-subjects analysis of variance (ANOVA) were conducted on the total time spent investigating objects during the familiarization phase, and on investigation time during the test. Pearson product-moment correlation coefficients were obtained between the total time spent investigating objects during familiarization and preference ratios obtained during the test phase. Correlations were analyzed separately by trial type.

4.2.6.2 DNMS task. A rat was considered to have made a correct choice when the novel object was displaced first during the test phase of each trial. During training, the percentage of trials with correct choices within each 4-trial session

was calculated. Average accuracy across the first 5 and final 5 training trials was obtained for each rat. For the test trials, accuracy was calculated as the percentage of trials with correct choices within each infusion condition (vehicle, E2, DPN), following each retention delay (0.5, 2, 3, & 5 min). These accuracy scores were determined from four trials for each trial type, with a total of 48 test trials**.** One-sample t-tests were conducted comparing the average accuracy scores obtained during the first 5, and last 5 sessions to chance level (50%). A dependent-sample t-test was also conducted on the change in accuracy across training. A two-way ANOVA was conducted on the accuracy scores obtained under each infusion condition following 3 retention delays (2, 3, and 5 min). Bonferroni-corrected planned comparisons were conducted comparing accuracy scores under each infusion condition following a 3-min and 5-min retention delay.

4.3 Results

4.3.1 Histology

Following all behavioural testing, rats received a lethal dose of sodium pentobarbital followed by transcardial perfusions with 0.9% saline (250 ml) followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (pH = 7.4, 250 ml). The brains were excised and stored in 4% paraformaldehyde solution for 4 hr before being transferred to a 30% sucrose/water solution overnight. The next day, the brains were transferred to a -80°C freezer until sectioning. Using a cryostat microtome, 40 µm coronal sections through the PRh were sliced and mounted on glass microscope slides. Sections were stained with Cresyl violet and histological analysis assessed cannula placement in rats used for the two

ORM tests separately. Analysis showed that three rats used for the DNMS task had improper cannulae placements and so their behavioural data was removed from the final analysis. The location of deepest cannula penetration in each hemisphere is presented in *Figure 4.1* for rats included in the NOP test and in *Figure 4.2* for those included in the DNMS task. A photomicrograph of a representative section is shown in *Figure 4.3* and indicates the extent of damage that occurred due to the cannula placement and multiple injections of vehicle, DPN, and E2.

4.3.2 NOP test

4.3.2.1 Familiarization phase. *Figure 4.4A* illustrates the total time spent investigating objects during the 5-min familiarization phase for each trial type (following vehicle, E2, and DPN infusions for trials with 4-hr and 72-hr retention delay). A two-way repeated-measures analysis of variance (ANOVA) revealed a main effect of retention delay, $F(1, 7) = 8.66$, $p = .02$, $\eta^2 = .55$, but no main effect of infusion condition, $F(2, 14) = 0.07$, $p = .94$, $\eta^2 = .01$, and no interaction, $F(2, 14) = 0.46$, $p = .64$, $\eta^2 = .06$.

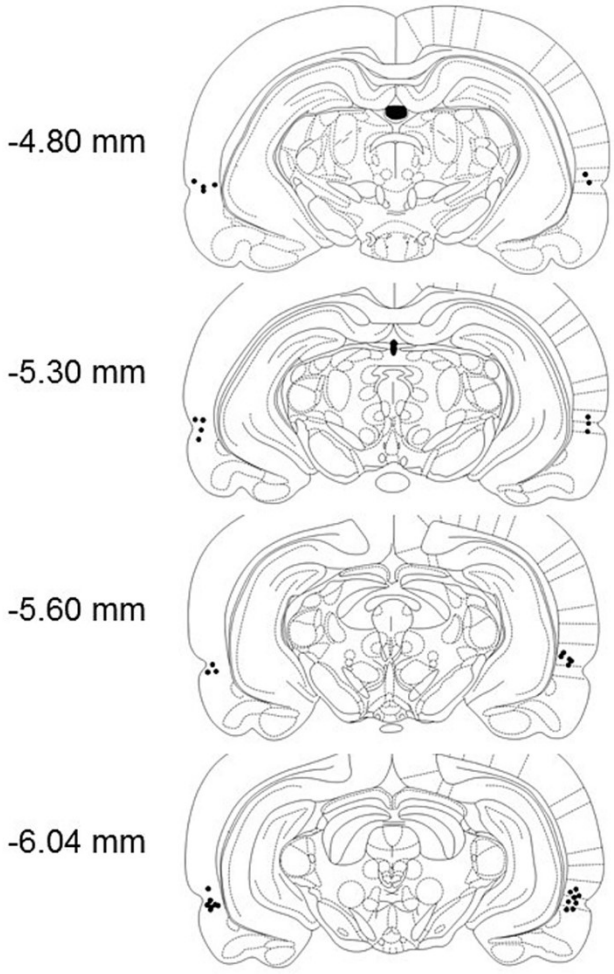

Figure 4.1: Schematic representation of the placement of cannula tips (indicated with black circles) in both hemispheres of rats tested on the NOP test (*n* = 10). Some tips are presented in multiple sections. Values indicate distance from Bregma (Paxinos and Watson, 1998).

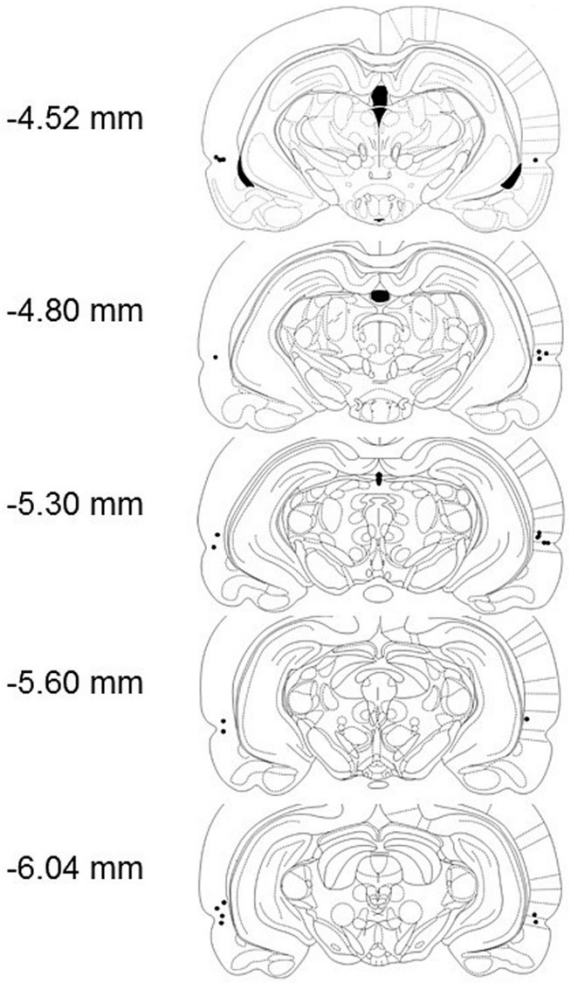

Figure 4.2: Schematic representation of bilateral placement of cannula tips of rats tested on the DNMS task (*n* = 7). Values indicate distance from Bregma (Paxinos and Watson, 1998).

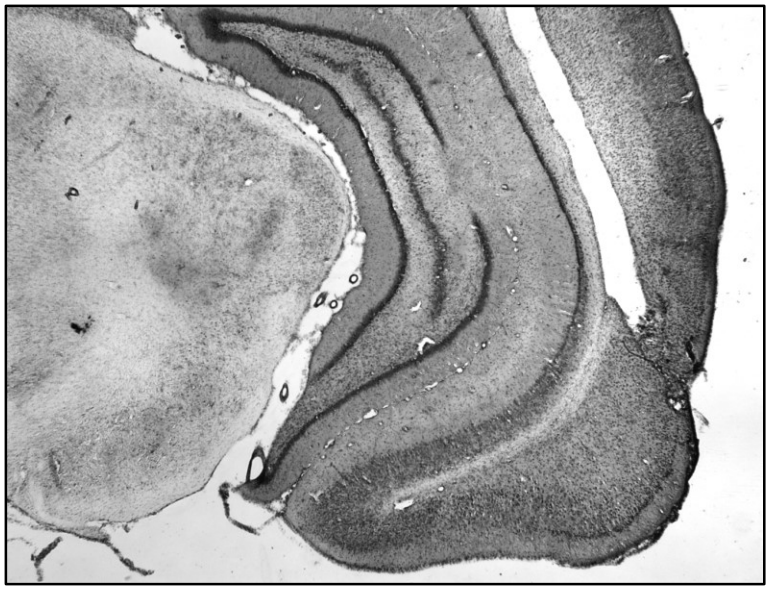

Figure 4.3. Photomicrograph of stained tissue depicting placement of a guide cannula in the PRh.

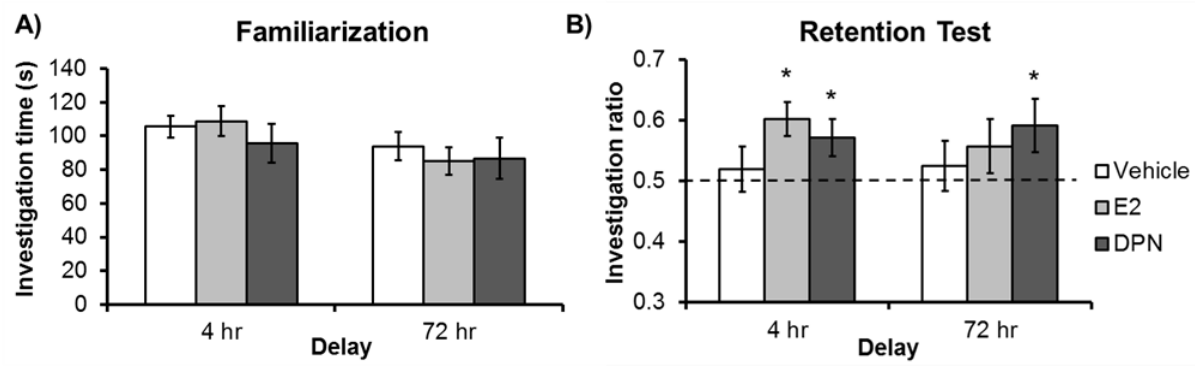

Figure 4.4. Object investigation during the NOP test following pre-familiarization infusions of DPN, E2 or vehicle. A. Mean time spent investigating the sample object during the 5-min familiarization phase of trials with a 4-hr and 72-hr retention delay. B. Investigation ratio obtained during the first 4 min of the 4-hr and 72-hr retention tests. (n = 10; * p < .05; one-sample t-test).

4.3.2.2 Test phase. *Figure 4.4B* presents the investigation ratios obtained for each trial type. One-sample t-tests (one-tailed) were conducted to compare performance during each trial type to chance (0.5). Investigation ratios that were statistically significantly above-chance occurred following E2, $t(8) = 3.42$, $p = .005$, Hedge's g = 1.13, and DPN infusions, $t(9) = 2.35$, $p = .02$, Hedge's g = 0.74 for trials with a 4-hr retention delay, and following DPN infusions, $t(9) = 2.05$, $p = .04$, Hedge's g = 0.65, for trials with a 72-hr delay. E2 infusions did not result in above-chance investigation ratios following a 72-hr retention delay, $t(9) = 1.28$, $p = .116$, Hedge's g = 0.41. Vehicle infusions resulted in no preference following either delay, 4 hr: $t(9) = 0.51$, $p = .315$, Hedge's g = 0.16; 72-hr: $t(8) = 0.56$, $p = .295$, Hedge's g = 0.19.

Total time spent investigating objects during the test phase was analyzed using a two-way repeated-measures ANOVA. No significant main effect of infusion condition, $F(2, 16) = 0.51$, $p = .61$, $\eta^2 = .06$, retention delay, $F(1, 8) = 2.81$, $p = .13$, $\eta^2 = .26$, and no interaction was found, $F(2, 16) = 1.73$, $p = .21$, $\eta^2 = .18$.

4.3.3 Correlations. Pearson correlations were conducted on the total time spent investigating objects during familiarization and the investigation ratios of the test for each of the 6 trials. None of the correlations were statistically significant, r = -.56-.03, p = .12-94.

4.3.4 DNMS task

Figure 4.5 presents performance during the first 5 and final 5 training sessions of the DNMS task. One-sample t-tests were conducted on performance at the beginning and at the end of training. Performance was no different from chance (50%) at the beginning of training (*M* = 49.06%, *SEM* = 4.17), *t*(15) = -0.23, *p* = .83, Hedge's g = -0.06, but was statistically significantly above chance at the end of training (*M* = 75.63%, *SEM* = 0.43), *t*(15) = 60.18, *p* = .000, Hedge's g = 15.00. In addition, a dependent-samples t-test revealed a statistically significant improvement in performance across training, *t*(15) = -6.23, *p* = .000, Hedge's g = -1.41.

Performance during the test under each infusion condition at each retention delay is presented in *Figure 4.6*. A two-way repeated-measures ANOVA revealed no main effect of infusion type, *F*(2, 12) = 0.36, *p* = .704, partial η^2 = .06, retention delay, *F*(2, 12) = 0.11, *p* = .895, partial η^2 = .02, or interaction, *F*(4,24) = 2.37, *p* = .081, partial η^2 = .28. Planned comparisons (Bonferroni- corrected) were conducted comparing performance of the different infusion conditions following 3-min and 5-min retention delays. Accuracy for the E2 infusion condition was statistically significantly lower than the vehicle condition following a retention delay of 5-min, *t*(6) = 5.16, *p* = .002, Hedge's g = 1.95. No other comparisons were statistically significant, t(6) = .00-.1.99, p = .094-1.0, Hedge's g = 0.00-2.27.

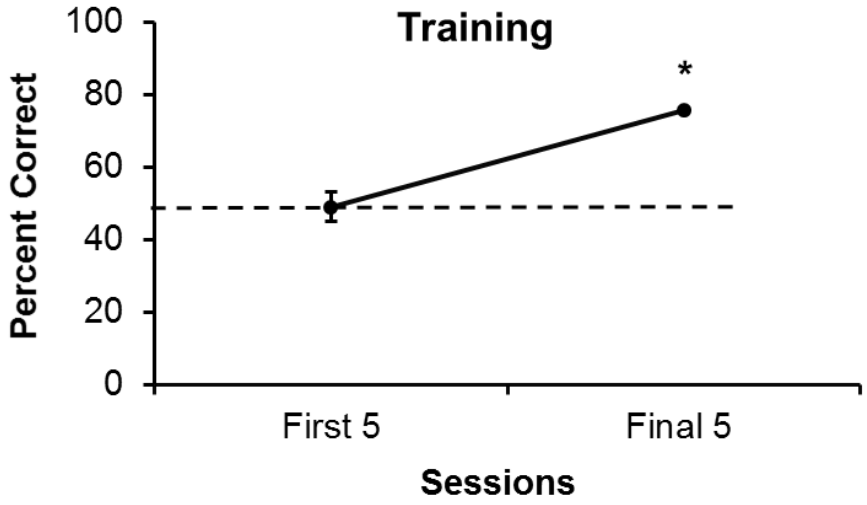

Figure 4.5. Percent trials correct during the training phase of the delayed nonmatch-to-sample task. Training trials displayed in two blocks of 5 sessions. The average percent correct for the first 5 sessions (first 20 trials) are at chance level (50%) and above chance for the final 5 sessions (last 20 trials; $n = 16$; $^*p < .05$; dependent-samples t-test).

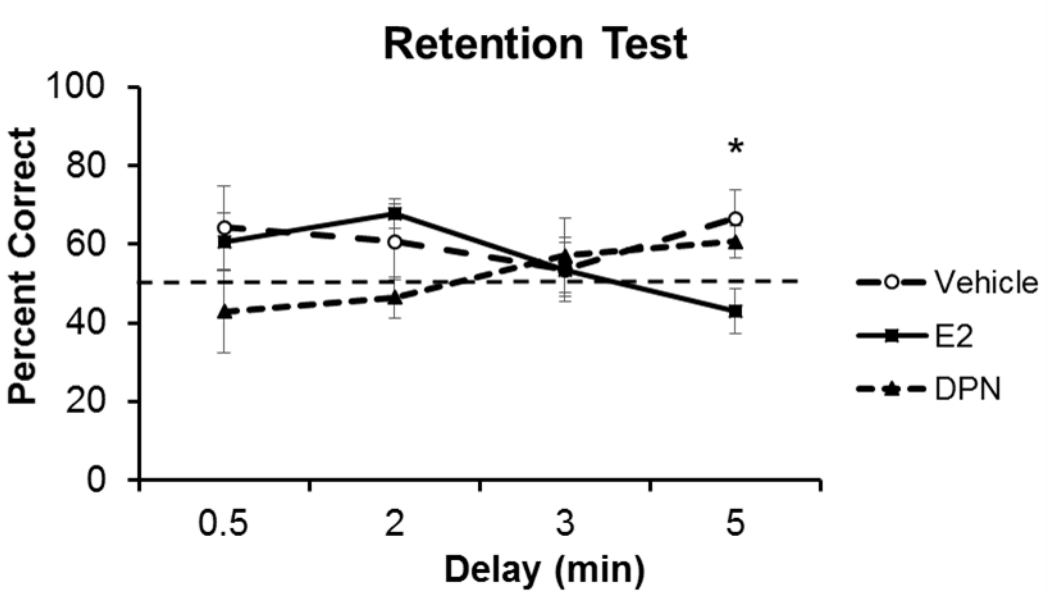

Figure 4.6. Performance during the test phase of the delayed non-match-to-sample task following infusions of DPN, E2 or vehicle. Percent trials correct across 4 retention delays. (n = 7; * p < .008; dependent-samples t-test).

4.4 Discussion

In the present study, DPN infused in the PRh before the familiarization phase of the NOP test resulted in novelty preference following a 4- and 72-hr retention delay. Although novelty preference was also observed following intra-PRh infusion of E2 following a 4-hr retention delay, no preference was observed following a 72-hr delay. Vehicle infusions resulted in a failure to demonstrate novelty preference regardless of delay. These results are consistent with the idea that E2 enhances novelty preference when it binds to ERβ in the PRh during familiarization. Previous studies also demonstrate enhanced novelty preference following systemic administration of DPN (rat: 3 mg/kg, s.c., Jacome et al., 2010; mice: 0.1 mg/kg, s.c., Walf et al., 2008), although one study reported no effect (30-150 μg/mouse, s.c.; Phan et al., 2011).

The results from the NOP test are consistent with the hypothesis that E2 enhances ORM via ERβ in the PRh. However, a failure to demonstrate novelty preference can occur with intact ORM. While the vehicle infusion did not result in an exploratory preference, rats in this condition may have still been able to recognize the sample object during the retention tests. Delay-dependent effects are consistent with treatment effects on memory ability. The enhanced novelty preference resulting from intra-PRh infusion of DPN was unaffected by retention delay, which suggests that E2 binding to ERβ in the PRh does not enhance ORM. Rather, E2 via ERβ may enhance non-cognitive processes, such as the rats' motivation to explore novelty. Consistent with this, enhanced novelty preference is observed following intra-HPC infusion of E2 (5 μg, Fernandez et al.,

2008) and theophylline, 8[(benzylthio)methyl]-(7Cl,8Cl)], an ERα antagonist (TPBM, 50 and 250 mg/µl, Pereira, Bastos, De Souza, Ribeiro, & Pereira, 2014) prevents novelty preference in OVX mice. Since ORM does not require the HPC (Mumby, 2001; Winters et al., 2008), such results do not likely reflect a manipulation on memory processes. In addition, previous studies have reported reinforcing properties of exploring novelty (e.g. Rebec, Christensen, Guerra, & Bardo 1997). Therefore, E2 binding at ERβ in the PRh or at ERα in the HPC may influence motivational processes that contribute to novelty preference without promoting ORM.

It is also possible that the range of retention delays used for the NOP test are ineffective at demonstrating a delay-dependent effect. An appropriate range of retention delays include those that are challenging and others that reliably result in the expression of novelty preference. Had a retention delay of only a few min been used, perhaps a delay-dependent effect would have been observed. However, this is unlikely to be the case, as Phan and colleagues (2012) reported enhanced novelty preference in OVX mice give E2 replacement (1.5-3 µg/kg, s.c.) following a 5-min retention delay.

While the lack of a delay-dependent effect is not entirely inconsistent with an effect on ORM, there is another factor to consider. Should performance on the NOP test reflect the strength of ORM ability, then spending more time exploring objects during familiarization should promote encoding of object representations. This would be reflected in a positive relationship between amount of time spent investigating the sample object during familiarization and performance on the

retention test. However, no correlation was found in the present study, and previous experiments also reported no such correlation (Gaskin et al., 2010; Gervais et al., 2013). Therefore, the lack of an association between performance during the familiarization and test phases is consistent with an enhancing effect of E2 on non-cognitive processes rather than ORM.

Results from the DNMS task also suggest that E2 does not enhance ORM. Unlike the NOP test, DPN had no effect on DNMS task performance, and E2 impaired performance following a 5-min retention delay, but not following any shorter delays. These results suggest that while E2 impairs DNMS task performance by acting within the PRh, it does not act via binding at ERβ. Intra-rhinal cortical infusion of E2 has previously been shown to impair DNMS task performance following a 3-min retention delay (Gervais et al., 2013). In contrast to studies that use the NOP test exclusively, the results of the present study and our previous study (Gervais et al., 2013) suggest that E2 impairs ORM by acting within the PRh. E2 does not appear to influence this ability by acting at ERβ, which is consistent with a previous study reporting no effect of DPN on DNMS task performance in macaques (Lacreuse et al., 2009). Taken together, the results suggest that E2 acts within the PRh to impair ORM, but not via ERβ.

Other ERs, including ERα (Shughrue et al., 1997) and the membrane-bound G-protein couple estrogen receptor (GPER1; Hazell et al., 2009) are expressed in the PRh. GPER1 is thought to be involved in many of the rapid (< 1 hr), non-genomic effects of E2, which include activation of second messenger cascades (i.e. cyclic AMP), protein kinases (PKA, CamKII) mitogen-activated

protein kinases (MAPK) and cAMP response element-binding protein (CREB; Korol & Gold, 2007; Spencer et al., 2008). A GPER1 agonist has been shown to produce some of these rapid effects of E2, including MAPK activation. It also increases Ca^{2+} mobilization and cAMP production (Prossnitz et al., 2008). Although there is some evidence suggesting that a GPER1 agonist enhances novelty preference (as reviewed by Ervin, Phan, Gabor, & Choleris, 2013), there is currently no published study investigating whether GPER1 influences DNMS task performance. There is also no published studies examining whether E2 binds to GPER1 in the PRh to impair ORM. Future studies should determine whether intra-PRh infusions of a GPER1 agonist impairs DNMS task performance in a delay-dependent manner.

The results of the present study support the idea that E2 binding at ERβ in the PRh enhances novelty preference without influencing DNMS task performance. This discrepant pattern of results is consistent with the interpretation that the enhanced novelty preference observed following intra-PRh infusion of E2 or an ERβ agonist reflects an influence on non-cognitive processes. Within the parameters used in the present study, the results from both tests suggest that while E2 acts within the PRh to impair ORM, when binding at ERβ, produces no discernible effects on this ability. It is currently unknown whether E2 binds to other ERs in the PRh to influence ORM. GPER1 is moderately expressed in this region and is known to produce rapid effects on cellular mechanisms implicated in memory (i.e. MAPK activation). Therefore,

future research is needed to determine whether E2 impairs ORM by binding at this receptor.

CHAPTER 5

Attenuation of synaptic density in the perirhinal cortex following 17-β estradiol replacement in the rat

Preface

Chapters 3 and 4 provide evidence that 17-β estradiol (E2) impairs ORM by acting within the perirhinal cortex (PRh). However it is currently unknown which neuronal correlates of learning and memory E2 influences in this region to modulate ORM. In the hippocampus (HPC), elevated levels of E2 are associated with increased excitatory synaptic transmission (Córdoba Montoya & Carrer, 1997; Wong & Moss, 1992), and increased synaptogenesis (Brake et al., 2001; Li et al., 2004; Woolley & McEwen, 1992, 1993). The majority of excitatory synapses form on spines located on dendritic branches (Yosihara et al., 2009), and so the density of these spines can reflect, indirectly, the density of excitatory (i.e. Glutamatergic) synapses. This increase is one proposed mechanism through which E2 is thought to enhance spatial and contextual learning and memory (Woolley, 2007).

Glutamatergic neurotransmission in the PRh is implicated in ORM. Although E2 modulates ORM via the PRh, it remains to be seen whether E2 also influences synaptic plasticity that may influence both glutamatergic neurotransmission and ORM. The purpose of the present study was to examine whether higher levels of E2 are associated with elevations in dendritic spine density in the PRh. Similar to Chapter 2, a35 and a36 were examined separately. The density of spines were compared under proestrous and estrous, as well as following high E2 replacement (10 μg/kg, s.c.) and no replacement in OVX rats. While no differences were found between the two naturally-cycling groups, high E2 replacement was associated with lower density of mature spines in a35.

Reduced synaptic density may decrease Glu neurotransmission, which might be a potential mechanism through which E2 influences ORM.

Abstract

Systemic and intra-perirhinal cortex (PRh) infusion of 17-β estradiol (E2) impair ORM (Gervais et al., 2013; Chapter 4). However, it is not currently known whether E2 influences synaptic plasticity in this structure. Higher E2 levels are associated with increased density of dendritic spines in the hippocampus (Woolley & McEwen, 1992, 1993), which form the majority of excitatory synapses in the central nervous system (Yosihara, De Roo, & Muller, 2009). The goal of the present study was to determine whether differences in dendritic spine density in the PRh are observed following endogenous changes in E2 levels and following E2 replacement in ovariectomized (OVX) rats. Density of total spines, and mature spines were compared between naturally-cycling rats in proestrous (n = 4) and estrous (n = 4), and also in ovariectomized rats given high E2 replacement (10 µg/kg, s.c.) and no replacement. The PRh is subdivided into Broadmann's area 35 and 36 and so group comparisons were made in each sub-region separately. No significant differences were found between proestrous and estrous in either sub-region of the PRh. However, high E2 replacement resulted in lower density of mature spines in a35 relative to no replacement. These findings suggest that higher E2 levels reduce synapse density in a35, which may result from spine shrinkage, or reduced synapse formation. This study provides some preliminary evidence for a mechanism through which E2 may impair ORM.

5.1 Introduction

Ovarian hormones, including 17-β estradiol (E2), modulate memory processes across species (Korol, 2004; Sherwin, 2012; Rapp, Morrison, & Roberts, 2003). For example, ovariectomized (OVX) rats given systemic (0.2 mg/kg, i.p.) or intra-hippocampal infusions of E2 (0.5 µg/µl), or vehicle were trained on a spatial memory (i.e. fixed-platform place navigation) task. The injections were given either immediately, or two hours following the final training trial. Systemic and local administration of E2 given immediately, but not 2-hr following training were associated with improved performance on a test given 24 hr later (Packard & Teather, 1997a,b). These studies as well as others (e.g. Korol & Kolo, 2002; Sinopoli, Floresco, & Galea, 2006) support the idea that E2 modulates spatial memory via actions within the hippocampus (HPC).

E2 also modulates excitatory synaptic transmission in the HPC. Higher E2 levels are associated with increased excitability (Wong & Moss, 1992), and a lower threshold necessary to induce long-term potentiation (Córdoba Montoya & Carrer, 1997) in the CA1 region of the HPC. This increase in excitability of CA1 neurons is proposed to result from an increase in the number of excitatory (i.e. glutamatergic) synapses in the region. Brake and colleagues (2001) demonstrated enhanced expression of three synaptic proteins, synaptophysin (a vesicular protein), syntaxin (located on pre-synaptic membrane), and spinophilin (post-synaptic protein located on dendritic spines) in the CA1 following high estrogen (E) replacement (estradiol benzoate, EB, 10 µg/kg, s.c.) relative to a no replacement condition. In addition to synaptic proteins, dendritic spines, which

are small protrusions on dendritic branches, are also sensitive to fluctuations in ovarian hormones, including E2. In the central nervous system, the majority of glutamatergic (Glu) excitatory synapses form on spines located on dendrites of postsynaptic neurons (Yosihara, De Roo, & Muller, 2009). Thus, a higher density of spines can reflect a great number of excitatory synapses, and so higher levels of E2 may be associated with a greater density of Glu synapses. Woolley and McEwen demonstrated higher density of dendritic spines on pyramidal neurons in the CA1 following proestrous relative to estrous (Woolley & McEwen, 1992), and following high E2 replacement relative to a no replacement condition (Woolley & McEwen, 1993). Taken together, the available data are consistent with an association between higher levels of E2 and increased density of excitatory synapses in the CA1, with some suggestion that this may lead to an increase in the excitability of this structure.

Although an association between E levels and dendritic spine density in the HPC has been replicated (e.g. Phan et al., 2012), one study (Li et al., 2004) using OVX mice reported no effect of E replacement (1 μg EB in 0.1 ml, s.c., for 5 days) on the density of all dendritic spine types. However, E replacement enhanced mushroom-shaped (i.e. mature) spines in the CA1 relative to a no replacement condition. In addition, both syntaxin and spinophilin in the dorsal HPC were enhanced by E replacement relative to no replacement. Mature spines have a well-defined neck and head and are believed to form synapses with other neurons (Matzusaki, 2007). Other spine types, including thin (i.e. immature) spines do not form active synapses (Matzusaki, 2007), but have the potential to

mature and form synapses later on (Li et al., 2004). Since there is no difference in the density of dendritic spines when immature ones were included, the findings from Li and colleagues (2004) suggest that E facilitates spine maturation, and perhaps synapse formation, given the enhanced expression of synaptic proteins. This maturation process may contribute to improved efficiency in excitatory synaptic transmission by E.

High E2 levels in the HPC are associated with enhanced spatial memory, but it does not necessarily enhance all memory abilities in all brain regions. For example, Korol and Kolo (2002) demonstrated that high E2 replacement (10 μg, in 0.1 ml oil, s.c.) in OVX rats impaired performance relative to a no replacement condition on a response-learning task, which is striatal-dependent. In addition, intra-striatal infusion of E2 (93.6 ng/0.5 μl) impaired performance of OVX rats on a response-learning task relative to vehicle infusion (Korol & Kolo, 2002). More recently, we (Gervais et al., 2013, Chapter 4) demonstrated that acute high E replacement (10 μg EB in 0.1 ml oil) or intra-perirhinal and entorhinal cortex infusion of E2 (244.8 pg/μl) impaired object-recognition memory (ORM) relative to a low E replacement (~22 pg/ml, serum) or a vehicle infusion condition.

Despite the lack of available evidence implicating the HPC in ORM (for a review, see Mumby, 2001), much of the research into the neuromodulatory mechanisms of this ability have focused exclusively on this structure. This extends to research investigating the modulatory effect of E2. Phan and colleagues (2011, 2012) demonstrate that, in OVX mice given low E2 replacement (1.5-3 μg.kg, s.c.) or the estrogen receptor (ER) α agonist (propyl

pyrazole triol, 50-75 μg, s.c.), novelty preference is enhanced and dendritic spine density in the CA1 is increased within 40 min of administration. The authors conclude that the enhanced novelty preference is the result of increased density of excitatory synapses in the CA1. While E2 may influence both novelty preference and dendritic spine density in the CA1, processes outside the HPC may be more specifically associated with novel-object preference and ORM.

All three known estrogen receptors (ERα, ERβ, GPER1) are expressed in the PRh (Blurton-Jones & Tuszynski, 2002; Brailoiu et al., 2007; Hazell et al., 2009; Shughrue, Lane, & Merchenthaler, 1997; Shughrue & Merchenthaler, 2001). Given that E2 may act in this region to impair ORM (Gervais et al., 2013; Chapter 4), perhaps E2 influences mechanisms within the PRh to modulate this ability. There is some evidence that E2 influences PRh function. For example, Fonseca and colleagues (2013) compared Fos-immunoreactive (IR) cells in the PRh following novel-object or open field exploration in OVX mice given chronic E2 replacement (0.18 mg/4 μl in corn oil, via silastic implant) or no replacement. Following exploration, the mice were perfused and Fos expression was examined. Fos is a protein marker for the c-fos IEG and a higher level of Fos-IR cells is taken to reflect more neuronal activity. Regardless of object exposure, E2 replacement resulted in more Fos-IR cells in the PRh, HPC, and amygdala. Within the E2 replacement condition, object exposure was associated with more Fos-IR cells in the PRh than the open field alone. The study by Fonseca and colleagues (2013) provides preliminary evidence that E2 influences PRh activity. In another study, acute high E2 replacement (10 μg in peanut oil, s.c.) decreases

$5HT_{1A}$ mRNA expression in the PRh (Osterlund & Hurd, 1998). Thus, there is some evidence that E2 influences both PRh function and ORM.

Glu is involved in synaptic transmission and plasticity in the PRh, including both long-term potentiation (LTP) and long-term depression (LTD; Kealy & Commins, 2011; Winters et al., 2008). LTP, which involves a persistent increase in response to high-frequency stimulation, is dependent on N-methyl-D-aspartate receptor (NMDAR; Winters et al., 2008). While LTD, which is a persistent decrease in response to low-frequency stimulation, is also dependent on NMDAR, it requires co-activation of metabotropic Glu receptors in the PRh (Brown & Bashir, 2002). Glu is implicated in ORM in rodents (Winters & Bussey, 2005b) and macaques (Matsuoka & Aigner, 1996; Ogura & Aigner, 1993), and pharmacological manipulations that prevent LTD in the PRh also prevent novelty preference in rats (Seoane, Massey, Keen, Bashir, & Brown, 2009; Warburton et al., 2003, 2005). Thus, LTD in the PRh is believed to be a potential neuronal mechanism for ORM (Winters et al., 2008; Brown, Barker, Aggleton, & Warburton, 2012). E2 is thought to modulate spatial and contextual memory via actions on synaptic plasticity in the HPC, including synaptic density, and so perhaps E2 may modulate ORM by influencing synaptic transmission and plasticity in the PRh. Since changes in synaptic density may reflect changes in Glu neurotransmission, any effect of E2 on synaptic density, or dendritic spine density, may result in alterations in Glu transmission.

The goal of the present study was to determine whether synaptic density (as inferred from measures of mature dendritic spine density) in the PRh is

influenced by E2 levels. Given evidence suggesting there are functional differences between Broadmann's area 35 (a35) and 36 (a36; Kealy & Commins, 2011), the density of spines in each sub-region was compared between proestrous (when E2 levels peak) and estrous (when E2 levels drop). Total spine density and density of mature spines were both examined. In addition, dendritic spine density in both areas following high E2 replacement or no replacement were also compared to determine the unique effect of E2.

5.2 Materials and methods

5.2.1 Subjects

Sixteen Long Evans female rats (125-150 g; Charles River, St Constant, Quebec) served as subjects. Upon arrival to the colony, rats were housed in pairs in transparent shoebox cages with woodchip and corncob bedding under 12:12 reverse light cycle (lights on at 8 pm). Male rats were present in the housing room. Rat chow and water was provided *ad libitum*. Approval for all procedures was provided by Concordia University's Animal Research Ethics Committee in accordance with the guidelines established by the Canadian Council on Animal Care.

5.2.2 Vaginal smear cytology

For half the rats (n = 8), daily vaginal smears were taken for a minimum of two weeks, 1-3 hr after lights off. Briefly, cotton-tipped applicators were used to gently swab the outer vagina wall to collect samples, which were then placed on microscope slides for evaluation. Samples were then viewed at 10x magnification using a Leitz Laborlux S microscope and were categorized as proestrous,

estrous, diestrous 1 or diestrous 2 based on the description provided by Marcondes, Bianchi, and Tanno (2002). Proestrous smears consist of predominately nucleated epithelial cells, estrous smears include anucleated cornified cells, diestrous 1 samples contain an equal proportion of all 3 cell types (epithelial, cornified, and leukocytes), and diestrous 2 samples consist of predominately leukocytes. All rats cycled every 4-5 days.

5.2.3 Surgery and hormone administration

The remaining eight rats were anesthetized using a cocktail (1 mg/kg, i.p.) of ketamine hydrobromide (50 mg/ml) and xylazine hydrochloride (4 mg/ml) prior to bi-lateral ovariectomy via a lumbar incision. Following surgical recovery, rats were maintained on estradiol (estradiol benzoate: 10 µg, Sigma-Aldrich, in 0.1 ml sesame oil, s.c.) and progesterone (500 µg, Sigma-Aldrich, in 0.1 ml sesame oil, s.c.), administered 2 days apart, until a washout period of two weeks that occurred prior to the start of the experiment. E2 replacement (10 µg/kg in sesame oil, s.c.) was given to four rats 48-, 24-, and 4-hr prior to being euthanized. The remaining four rats received the oil control condition (0.2 ml/kg sesame oil, s.c.; n = 4) at the same injections times as the E2 replacement condition.

5.2.4 Perfusion and Golgi-Cox impregnation

On the evening (1.5-3 hr after lights on) of either proestrous (n = 4) or estrous (n = 4), or 4 hr following the third injection of E2 (n = 4) or oil (n = 4), rats received a lethal dose of sodium pentobarbital before receiving a transcardial perfusion with 0.9% saline (400 ml). Brains were excised, and cut to a length of

approximately 10 mm before being immersed in an impregnation solution (~5 ml) containing mercuric chloride, potassium dichromate, and potassium chromate (Rapid GolgiStain Kit, FD NeuroTechnologies). The brains were then stored in the dark at room temperature for a two-week period. The impregnation solution was changed 24 hr following perfusion. Following the impregnation period, the tissue was transferred to a sucrose solution and stored at 4°C in the dark between 2-7 days. Using a cryostat, 100-µm coronal sections that included the PRh were taken and blotted onto gel-coated microscope slides. Sections dried naturally in the dark for 1-3 days before being rinsed, stained with an ammonium hydroxide film fixative solution, and then dehydrated and cover slipped with Permount (Sigma, Canada). The slides were stored in the dark for approximately 6 months before microscopic analysis occurred.

5.2.5 Microscopic analysis

Dendritic spine density analysis was conducted using Neurolucida software (version 7, MBF BioScience) on a PC computer, which was connected to a Hamamatsu ORCA-ER camera mounted on a Leica DM 5000 B microscope. For each rat, six neurons (3 in a35 and 3 in a36) in the anterior (2.56-4.16 mm from Bregma), middle (4.30-6.04 mm from Bregma), and posterior (6.30-6.72 mm from Bregma) portions of the PRh were chosen for analysis. The borders of a35 and a36 are based on Burwell (2001). Neurons were selected for analysis using the following criteria: a) pyramidal shape, categorized by one of 5 types described by Furtak, Russell, and Brown (2007) b) fully impregnated, and c) relatively isolated from neighbouring cells to prevent selecting dendrites for

analysis from a different neuron. Once a neuron was selected, two dendritic branches were selected for analysis. Selected branches were a) not truncated, b) not a primary dendrite, and c) were completely visible. Whenever possible, the two selected branches came from a different section of the neuron, and varied in length.

Using Neurolucida, each dendritic branch was traced twice at 400x magnification. The first trace included every spine type visible on the branch. The second trace included mature, mushroom-shaped spines only, which are spines with well-defined head and neck structures (as described in Li et al., 2004). The experimenter tracing each branch was blind to group membership. Dendritic spine densities were obtained for each branch for all spine types, and for mature spines only. Densities obtained from the two branches per neuron were averaged separately for total spine type and mature spine, then averages across the three neurons in a35 and the three in a36 were calculated for each rat. In other words, a total of four density scores were obtained for each rat, 1) density of total spines in a35, 2) density of total spines in a36, 3) density of mature spines in a35, and 4) density of mature spines in a36. Statistical comparisons were made between proestrous and estrous groups, and between E2 replacement and no replacement for each of the four density scores obtained.

5.3 Results

5.3.1 Spine density in cycling rats

Figure 5.1A presents the total number of spines on dendritic branches in a35 and a36 under estrous and proestrous. Independent-samples t-tests were

conducted comparing the density of all spine types in each area. No statistically significant group differences were found, a35: $t(6) = -0.08$, $p = .99$, Hedge's g = -0.06, a36: $t(6) = -0.77$, $p = .469$, Hedge's g = -0.54.

The number of mature spines per 10 µm under estrous and proestrous is presented in *Figure 5.1B*. An independent-samples t-test was conducted comparing the density of mature spines in a35 and a36 under estrous and proestrous. No statistically significant group differences were found, a35: $t(6) = 2.26$, $p = .065$, Hedge's g = 1.60, a36: $t(6) = 0.95$, $p = .378$, Hedge's g = 0.67.

5.3.2 Spine density in OVX rats with and without E2 replacement

The total number of spines per 10 µm in both sub-regions of the PRh is presented separately for each group in *Figure 5.2A*. An independent-samples t-test was conducted comparing the density counts of each group in each area. No statistically significant group difference was found in either area, a35: $t(6) = -1.13$, $p = .303$, Hedge's g = -0.80, a36: $t(6) = -0.97$, $p = .371$, Hedge's g = -0.68.

Figure 5.2B presents the density of mature spines in a35 and a36 is presented separately by group. Two independent-samples t-tests were conducted on each sub-region and a statistically significant differences was found for a35, $t(6) = 3.43$, $p = .014$, Hedge's g = 2.42. No difference was found for a36, $t(6) = -0.05$, $p = .961$, Hedge's g = -0.04. *Figure 5.3* presents photomicrographs of representative dendritic branches in a35 following E2 replacement and no replacement.

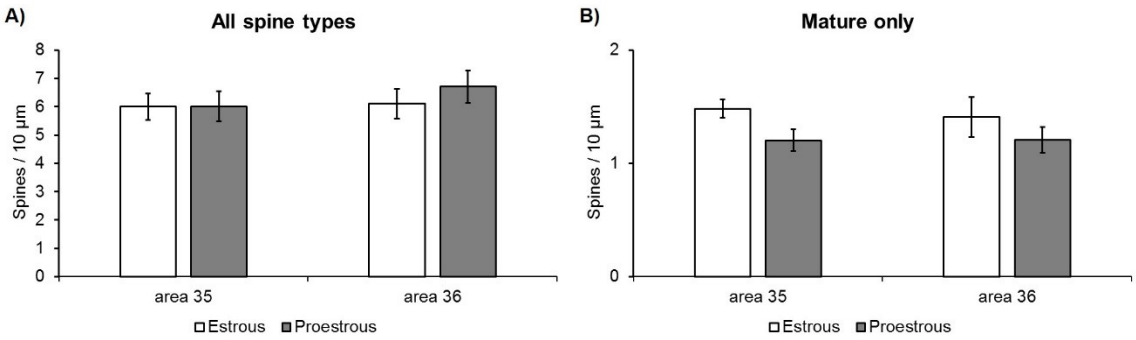

Figure 5.1. Dendritic spine density in a35 and a36 under proestrous (*n* = 4) and estrous (*n* = 4). A. Total number of dendritic spines per 10 µm for each group. B. Number of mushroom-shaped spines per 10 µm for each group.

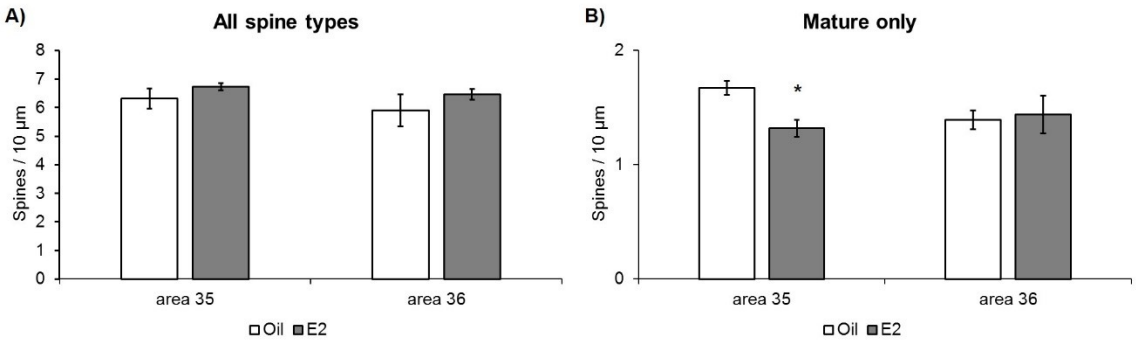

Figure 5.2. Dendritic spine density in a35 and a36 following E2 replacement (10 µg/kg, s.c.; *n* = 4) and no replacement (*n* = 4). A. Total number of dendritic spines per 10 µm for each group. B. Number of mushroom-shaped spines per 10 µm for each group. (*$p < .05$; independent-samples t-test).

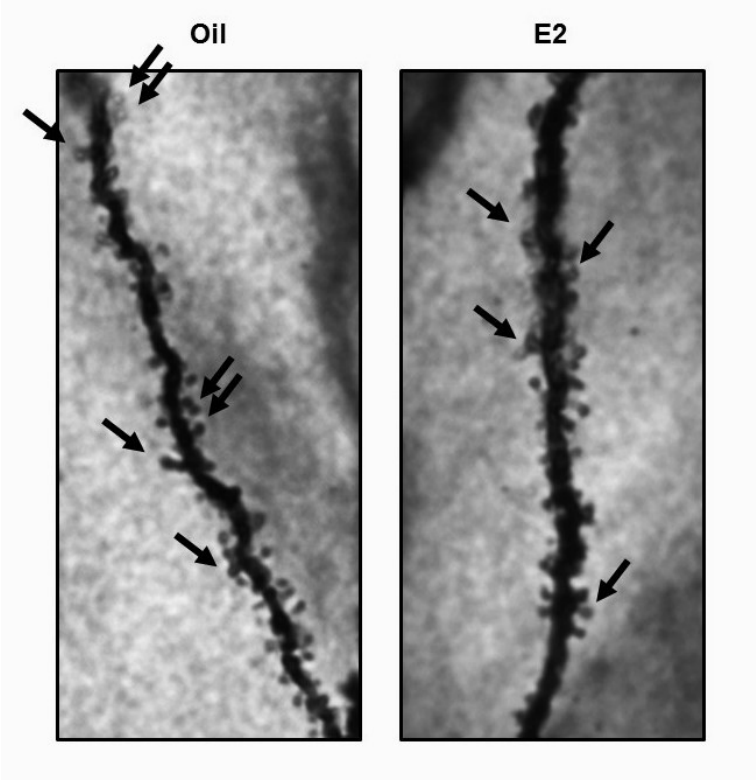

Figure 5.3. Photomicrographs of representative dendritic branches in a35 following E2 replacement and no replacement. Images were taken at 40x and illustrate the number of visible spines at one section of a branch. Mature spines are indicated with arrows.

5.4 Discussion

Natural fluctuations in ovarian hormones (including E2) did not influence dendritic spine density in the a35 and a36 of the PRh. This was true for the total number of spines and for mature spines. Although natural fluctuations in ovarian hormones appear to influence the density of dendritic spines in CA1 (e.g. Woolley & McEwen, 1992), the results from the present study suggest this is not the case in the PRh.

In contrast to the results from the cycling rats, high E2 replacement (10 μg/kg, s.c.) in OVX rats was associated with lower density of mature spines on dendritic branches in a35 relative to no a E2 replacement group. There were no group differences in the density of total spines in either sub-region, nor in the density of mature spines in a36. High E2 replacement has previously been shown to increase dendritic spine density in the CA1 (Phan et al., 2012; Woolley & McEwen, 1992, 1993), ventromedial hypothalamus (Segarra & McEwen, 1991), and infralimbic/prelimbic prefrontal cortex (Velasquez-Zamora, Garcia-Segura, & Gonzalez-Burgos, 2012). The density of mature spines in the CA1 has been shown to increase, or not change following E2 replacement or administration of an ERβ agonist (Li et al., 2004; Liu et al., 2008; Mukai et al., 2007). Therefore, the direction of the effect observed in the PRh is opposite to the effect of high E2 observed in other brain regions. Taken together, high E2 levels appear to influence synapse density, but the direction of the effect depends on the brain region being examined.

Mature spines are thought to form excitatory synapses with other neurons (Li et al., 2004). Therefore, the observed decrease in density of mature spines may reflect a reduction in the density of excitatory synapses in a35. This decrease in mature spine density without an effect on all spine types is consistent with spine shrinkage (rather than spine elimination). Spine shrinkage, which consists of transforming large, mature spine, into smaller ones, has been shown to occur following low-frequency stimulation that induces LTD (Matzusaki, 2007). Spine shrinkage can transform the spine into an inactive one, but can also result in an active spine with a smaller post-synaptic density, and fewer AMPAR and NMDAR (Matzusaki, 2007). Since AMPAR are implicated in baseline excitatory transmission and NMDAR in synaptic plasticity, it seems possible that spine shrinkage can decrease excitatory transmission. Alternatively, it is possible that the reduced density of mature spines is the result of reduced spine maturation, or synapse formation. Synapse formation, which involves recruiting AMPAR and NMDAR, has been shown to occur following LTP (Matsuzaki et al., 2007), and reducing synapse formation will prevent increases in excitatory transmission. The parameters used in the present study do not allow for conclusions to be made regarding which mechanism is responsible for the reduced density of synapses. Therefore, although the results support the idea that high E2 replacement reduces excitatory transmission in a35, future research is needed to determine whether this results from spine shrinkage, or reduced synapse formation.

NMDAR antagonism in the PRh prevents novelty preference (Winters & Bussey, 2005b) and impairs DNMS task performance (Matsuoka & Aigner, 1996; Ogura & Aigner, 1993). Systemic and intra-PRh infusion of E2 (Gervais et al., 2013, Chapter 4) also influences novelty preference and DNMS task performance. Thus, high E2 replacement appears to influence both PRh synaptic density and ORM. It is important to determine whether both effects are related and if so, whether E2 influences ORM via reduced density of mature spines in a35.

Although the effects of E2 on dendritic spines in the CA1 appear to be rapid, with a higher density of spines being observed within 40 min of administration (Phan et al., 2012), the results of the present study suggest the effect in the PRh is slow, requiring more than 24 hr. The estrous phase of the rat estrous cycle typically begins 24 hr after proestrous. Thus, any fluctuations in dendritic spine density between these two phases have to occur within that time frame. No statistical difference were observed between these two phases in the present study, but differences were observed between the high E2 replacement and no replacement groups. Since E2 was administered 48, 24, and 4 hr before the rats were perfused, the observed decrease in mature spines appears to result from mechanisms occurring within this two-day time frame. Genomic actions of E2 are thought take hours to days (Vasudevan & Pfaff, 2008), and be mediated by changes in gene expression following ligand binding to ERs. These changes will either promote or inhibit protein expression (Nelson, 2005). As inferred by the results of Phan and colleagues (2012), spine development

appears to be rapid. However, the time course for morphological changes, including spine shrinkage and synapse formation, is less clear. Thus, it is possible that such transformations require more than 24 hr.

Although no statistical difference was observed in the density of mature spines between proestrous and estrous, an examination of the effect size estimates (i.e. Hedge's g) suggests the density in a35 may be different between proestrous and estrous. The density of mature spines is 1.6 SD greater under estrous than proestrous, which is a large difference. The lack of a statistical difference between the two groups may be the result of insufficient power resulting from a small sample size (*n* = 4/group). Although the majority of studies investigating dendritic spine density in the HPC use similar group sizes (*n* = 4-5, except Li et al., 2004: *n* = 10), it is possible larger groups are required to detect differences in mature spines in the PRh. Since the effect size estimates comparing mature spines in a35 are large for both samples (Hedge's g = 1.6 between proestrous & estrous; Hedge's g = 2.42 between high E2 replacement and no replacement), it is possible that spine shrinkage or reduced synapse formation in a35 resulting from higher levels of E2 occurs rapidly.

Another possibility is that circulating levels of progesterone may influence mature spines, masking any effect of E2 during the estrous cycle. During proestrous, both hormones peak, albeit at different times during this phase. During estrous, E2 levels reach nadir, and although progesterone levels also decrease, they are more stable and do not drop to their lowest point (Hussain, Shams, & Brake, 2014). Progesterone (20 mg/kg, i.p.) prevents the enhancing

effects of E2 (0.2 mg/kg, i.p.) on a spatial memory task in OVX mice (Harburger, Bennett, & Frick, 2007). Although there is some evidence that progesterone counteracts the enhancing effects of E2 on spatial memory, progesterone appears to enhance the effect of E2 on dendritic spine density in the HPC (Gould, Woolley, Frankfurt, & McEwen, 1990). This is substantiated by evidence that fluctuations in dendritic spine density during the estrous cycle corresponds to the differences observed between E2 replacement and no replacement (Woolley, 1998). Although progesterone appears to enhance, and not attenuate the effects of E2 on dendritic spine density in the HPC, the opposite may be true in the PRh. Future research is needed to explore this possibility.

One final explanation for why higher levels of E2 during proestrous may not have produced observable effects on mature spine density concerns the level of circulating E2 in the control group. During estrous, circulating levels of E2 are low, but present (~20 pg/ml serum, Haim et al., 2003), whereas very low levels of E2 are present following OVX (~6.5 pg/ml, serum; Almey, Hafez, Hantson, & Brake, 2013). Given that the high E2 replacement used in the present study has previously been shown to produce serum concentration levels comparable to proestrous (Quinlan, Hussain, & Brake, 2008), the difference in E2 levels between the high E2 replacement and no replacement groups is greater than the difference between proestrous and estrous. Thus, it is possible that the fluctuations in E2 levels across the estrous cycle are not sufficient to produce observable differences in mature spine density. Future studies are needed with larger group sizes to determine whether this is the case. In addition, determining

whether spine shrinkage or reduced synapse formation in a35 occurs following higher levels of E2, and whether progesterone enhances or attenuates the effect of E2 are important issues to address.

High E2 replacement reduces the density of mature spines in a35, but has no effect in a36. Although the functional differences between these two sub-regions is unknown, there is anatomical evidence suggesting they are distinct (for a review, see Kealy & Commins, 2011). For example, the majority of the connections between the lateral entorhinal cortex and PRh involves the rostral portion of a35 (Burwell & Amaral, 1998). However, as suggested by Kealy and Commins (2011), too few studies investigating the PRh have taken this differentiation into consideration. The results from Chapter 2 suggest some level of functional difference between a35 and a36 in novelty-related neuronal activation. However, more research distinguishing the role of these two sub-regions in ORM is warranted.

The results of the present study demonstrate that high E2 replacement in OVX rats reduces synaptic density in a35 of the PRh. This suggests that higher levels of E2 are associated with spine shrinkage or reduced synapse formation in this region, which may result in reduced excitatory neurotransmission. This reduction might explain the observed effect of intra-PRh infusion of E2 on ORM (Gervais et al., 2013; Chapter 4). An important next step concerns examining whether decreases in mature spine density in a35 following E2 replacement are associated with impaired ORM.

CHAPTER 6

General Discussion

6.1 Summary of findings

The purpose of this thesis was to investigate whether ACh and E2 affect object-recognition memory (ORM) by influencing neuronal function and synaptic plasticity in the PRh, a structure important for this type of memory. To determine if they act within the PRh, bi-lateral cannulae were chronically implanted in this region (in Chapters 2-4) allowing for multiple local infusions. An mAChR antagonist (atropine sulfate, 100 μM) was used in Chapter 2, water-soluble E2 (244.8 pg/μl) in Chapter 3, and an agonist of ERβ (DPN; 2 μg/μl) in Chapter 4 and their effects on ORM was assessed using the NOP test (Chapters 2-4) and DNMS task (3-4). Infusions occurred either immediately before (Chapters 2-4), immediately after (Chapter 2-3), or 2 hr following (Chapter 3) the familiarization phase of the NOP test to determine whether ACh and E2 play a role during familiarization, or consolidation. A 4-hr (Chapters 2 & 4), 24-hr (Chapters 2), and 72-hr (Chapter 4) retention delay was used to observe whether any effect on novelty preference is delay-dependent. The results from Chapters 3-4 suggest that E2 binding to ERβ in the PRh enhances novelty preference (regardless of retention delay). Furthermore, Chapter 2 suggests that ACh binding to mAChR also enhances novelty preference when tested 4- and 24-hr later. When performance was assessed on the DNMS task in Chapters 3-4, E2 infusions had no effect on performance at retention delays of 0.5 and 2 min, whereas performance was significantly impaired at delays of 3 min (Chapter 3) and 5 min (Chapter 4). Thus, the same treatment that promoted novelty-preference produced delay-dependent deficits on the DNMS task. Taken together, the

results demonstrate that ACh (via mAChR) and E2 (via ERβ) influences novelty preference and that E2 (independent of ERβ) impairs DNMS task performance. These results are consistent with a modulatory effect of ACh and E2 in ORM and suggest they influence processes in the PRh that promote memory formation.

Chapter 2 demonstrates that neuronal activation in the posterior portion of a36 (as measured by Fos-IR) increases following novel-object exploration. This increase is mediated by ACh binding to mAChR in the PRh, suggesting a modulatory effect of this neurotransmitter in novelty-related neuronal activation. The dendritic spine analysis in Chapter 5 reveals that high E2 replacement reduces synaptic density in a35 of the PRh. This final study provides preliminary support for a modulatory effect of E2 on synaptic plasticity in the PRh. Taken together, the four studies described in this thesis support a modulatory effect of ACh and E2 in ORM, neuronal activation and synaptic plasticity in the PRh.

6.2 Theoretical Implications

6.2.1 Mechanisms of ORM

Reduced firing of PRh neurons that occurs following presentation of familiar relative to novel stimuli is thought to reflect a neuronal mechanism for familiarity discrimination (Brown & Aggleton, 2001; Zhu, Brown, & Aggleton, 1995). Since ACh is implicated in the acquisition of spatial memory (Marrosu et al., 1995; Sutherland, Whishaw, & Regehr, 1982), Chapter 2 addressed whether ACh binding to mAChR promotes the initial encoding that is thought to occur following object exploration. Fos-immunoreactivity (IR) was used as a measure of neuronal activation in the PRh. Not only was there reduced Fos-IR following

familiar relative to novel-object exploration, this difference was prevented by a mAChR antagonist, which suggests this receptor type might play a role in novelty-related neuronal activation. LTD promotes IEG expression (including c-fos; Lindecke et al., 2006), and so the observed effect of mAChR in Chapter 2 on neuronal activation within the PRh may result from effects on this type of synaptic plasticity. The results from the present thesis are consistent with a modulatory effect of ACh (via mAChR) in PRh synaptic plasticity, with potential implications for ORM. Although it is believed that PRh synaptic plasticity contributes to ORM abilities (for a review, see Winters et al., 2008), few studies have addressed this directly. An important next step is to determine whether neuroplastic changes resulting from ACh binding to mAChR influence performance on the NOP test and DNMS task.

Chapter 5 demonstrates that higher E2 levels are associated with lower synaptic density in a35 of the PRh. These results suggest that high levels of E2 may decrease glutamatergic neurotransmission in the PRh, via spine shrinkage or reduced spine formation, both of which reduce expression of AMPAR and NMDAR. This is an important finding, as it provides preliminary evidence for how E2 might modulate ORM. Previously, Phan and colleagues (2012) demonstrated that E2 replacement (1.5-3 μg/kg, s.c.) enhanced novelty preference and increased density of dendritic spines in the CA1 within 40 min of administration to OVX mice. The authors suggested that E2 mediates ORM via changes in synaptic density in the CA1. While the increased synaptic density reported by Phan and colleagues may certainly reflect a potential mechanism through which

E2 influences HPC-dependent learning and memory, this does not likely extend to ORM. As described in Section 1.3-1.4 in the introduction to this thesis, there are several studies done in macaques and rodents that demonstrate the HPC is not involved in ORM. So, any neuroplastic changes occurring within this structure are not expected to influence ORM. While there is evidence that E2 modulates ORM, any suggestion that E2 improves ORM via increased synaptic density in the CA1 is likely false. Therefore, in order for E2 to modulate ORM, it must do so in other brain structures.

Unlike the HPC, the PRh has received strong support for a role in ORM (see Sections 1.2-1.4 of the introduction). Chapters 3 and 4 of the present thesis provide evidence that E2 impairs ORM via actions in this structure. Morphological changes in PRh neurons are also observed following E2 replacement, as was described in Chapter 5. Although changes in dendritic spine density are evident in both the HPC and PRh following E2 replacement, it seems more likely that the changes observed in the PRh underlie the modulatory effect of E2 on ORM than those in the HPC. An important next step is to determine whether decreased synaptic density in the PRh resulting from higher E2 levels influences ORM.

Taken together, the results presented in this thesis provide evidence that both ACh and E2 modulate ORM and PRh synaptic plasticity. The following two sections discuss the results of both ORM tests in terms of the proposed role of ACh and E2.

6.2.2 Role of ACh in novelty preference or ORM

The majority of previous studies investigating the role of mAChR in ORM used the NOP test. The findings from these studies suggest that ACh binding to this receptor during familiarization promotes novelty preference on a test given within one hour. Three questions remain about its role. One concerns whether ACh binding to mAChR influences novelty preference following longer retention delays (> 1 hr). There are several studies demonstrating that mAChR antagonists prevent novelty preference on test given within 60 min of the familiarization phase (Bartolini et al., 1996; Ennaceur & Meliani, 1992; Pitsikas et al., 2001; Vannucchi et al., 1997; Warburton et al., 2003). In addition, Tinsley and colleagues (2011) demonstrated that mAChR antagonism prevents novelty preference following a 20-min but not 24-hr retention delay. This suggests that ACh binding to mAChR may not influence long-term ORM. However, it is also possible that mAChR antagonism influences performance during the test phase. The half-life of scopolamine is 4.5 hr and so the drug is likely still active on a test given within this time frame, which was the case for the 20-min, but not 24-hr, retention test in the Tinsley and colleagues (2011) study. Although Winters and colleagues (2006) demonstrated that intra-PRh infusion of scopolamine given immediately before a 24-hr retention test has no effect on the magnitude of the novelty preference, more research is needed to examine whether mAChR antagonism prevents novelty preference following retention delays longer than 1 hr. In Chapter 2, intra-PRh infusion of atropine sulfate (100 μM), which has a half-life of 3 hr, was given immediately before the NOP test trial with a retention delay of 4 hr or 24 hr. This allowed for an examination of mAChR antagonism

during familiarization on novelty preference following retention delays longer than those used previously. The intra-PRh infusions of atropine prevented novelty preference following both retention delays, and so the findings are consistent with previous studies that used shorter delays. Taken together, mAChR antagonism during familiarization appears to influence novelty preference following delays varying from 15 min to 24 hr.

A second question concerns whether the effects of mAChR antagonism on novelty preference reflect a role of ACh (via mAChR) in ORM, or non-cognitive processes that influence the expression of a preference. Although not entirely eliminating this issue, NOP test trials with retention delays that vary considerably in their length can be used to determine whether mAChR antagonism is delay-dependent. The shortest retention delay should be brief enough to reliably result in novelty preference. The longest delay should be long enough to barely result in above-chance novelty preference. If mAChR antagonism influences long-term ORM, then intra-PRh infusions of atropine should prevent novelty preference following the long delay, but novelty preference should be intact in both groups at the short delay. Tinsley and colleagues (2011) demonstrated that mAChR antagonism in the PRh prevented novelty preference following a 20-min, but not 24-hr retention delay. This pattern is consistent with an effect on short-term ORM. In the present study, mAChR antagonism prevented novelty preference following both a 4-hr and 24-hr retention delay. Since no delay-dependent effect was found, it is entirely possible that mAChR antagonism influences non-cognitive processes that effect novelty

preference. These processes might include the motivation to explore novelty. Rebec and colleagues (1997) suggest there are reinforcing properties of exploring novelty. So, any treatment that manipulates novelty preference may alter whether novel-object exploration is reinforcing. Although the lack of a delay-dependent effect is consistent with such an effect, it does not entirely rule out a role of ACh (via mAChR) in ORM, as it may influence both short- and long-term memory. Incorporating the DNMS task in future studies will help clarify whether mAChR antagonism influences ORM or motivational processes that influence the expression of novelty preference.

The third question concerns whether ACh also promotes novelty preference by binding to mAChR following familiarization. Such an effect is consistent with a role in the consolidation of object representations. Chapter 2 describes evidence that blocking ACh binding to mAChR prior to familiarization prevents novelty preference following two retention delays (4 & 24 hr), and that blocking ACh binding following familiarization also prevents novelty preference. The NOP test results from Chapter 2 support the idea that ACh modulates novelty preference via mAChR in the PRh during or immediately following exploration of familiar objects. However, previous studies demonstrated either no effect (Warburton et al., 2003), or enhanced novelty preference (Winters et al., 2006) when mAChR antagonists are administered following familiarization. Given the inconsistent pattern of results, future studies are needed to confirm whether mAChR antagonism during consolidation prevents novelty preference.

As discussed in Section 6.2.5 (below), novelty preference can be prevented despite intact ORM. Therefore, it is necessary to rule out non-cognitive effects when attempting to understand any treatment effect on the expression of novelty preference. Determining whether ACh binding to mAChR produces delay-dependent effects using more varied retention intervals on the NOP test and DNMS task is a crucial next step towards understanding the role of ACh (via mAChR) in ORM.

6.2.3 Role of E2 in ORM

Previous studies demonstrate that high E2 replacement in OVX rats facilitates novelty preference (Fernandez & Frick, 2004; Gresack & Frick, 2006; Inagaki, Gautreaux, & Luine, 2010; Luine, Jacome, & MacLusky, 2003). Two major concerns about conclusions made based on these studies were addressed in this thesis. One concerns whether the enhanced novelty preference reflects improved ORM abilities. The second concerns which regions E2 affects to modulate ORM. Chapters 3 and 4 were designed to address both concerns. The DNMS task was used in conjunction with the NOP test, the latter of which is the more commonly used ORM test. Although previous studies investigated intra-HPC effects on novelty preference (Fernandez et al., 2008), the present thesis focused on the PRh. The results demonstrate a striking difference between the modulatory effect of E2 on novelty preference and DNMS task performance. E2 enhanced novelty preference via ERβ in the PRh regardless of retention delay, and impaired DNMS task performance (in the PRh, but independent of ERβ) in a delay-dependent manner. Not only does intra-PRh E2 appear to have divergent

effects on two ORM tests, it appears to influence them via binding to different ERs.

Determining whether E2 modulates ORM in rodents has important implications for research into the modulatory effect of this hormone on cognition in women. Decreases in endogenous levels of estrogens, including E2, is implicated in cognitive impairments observed following pregnancy and following menopause (Sherwin, 2012). Although the results from the present study indicate that higher levels of E2 are associated with deficits in cognitive function, which is opposite to the pattern observed in women, a number of factors can influence the direction of results. These include age of animals, dose, duration of treatment, mode of delivery, abilities required for successful task completion, motivation, sex, stress, interaction with other hormones (Korol, 2004; Zurkovsky, Brown, Boyd, Fell, & Korol, 2007). The majority of studies investigating the modulatory effect of E2 on cognitive ability compare OVX animals with E2 replacement to those without replacement. In Chapters 3-4, rats were ovariectomized and given chronic low E2 replacement, with either acute high E replacement, or intra-PRh infusion of E2 or vehicle. In other words, rats in the control condition had circulating levels of E2 that were low, but greater than control animals used in previous investigations. This difference in circulating levels of E2 in the control condition might explain the different pattern of results. Future investigations are needed to determine whether the dose and duration of hormone replacement influences how E2 modulates ORM and PRh synaptic plasticity. Additional experiments such as these will provide important information as to whether

higher E2 levels are expected to improve or impair visual recognition memory in women.

6.2.4 Interpretational challenges to the NOP test when used as the sole test of ORM

As stated earlier in Section 6.2.4, novelty preference can be prevented for reasons other than impaired ORM. Therefore, understanding why a treatment group fails to demonstrate novelty preference becomes challenging in the absence of any other ORM test. Although it does not entirely eliminate this shortcoming, some researchers incorporate two NOP test trials with different retention delays to determine whether treatment effects are delay-dependent. Effects that are believed to influence long-term memory processes are predicted to influence performance following longer compared to shorter retention delays as they are more cognitively taxing. This approach was used in Chapter 2 and 4, with a shorter retention delay of 4 hr and longer retention delay of 24 hr for Chapter 2, and 72 hr for Chapter 4. In both chapters, the treatment effects was unaffected by retention delay.

An additional attempt was made in Chapters 2 and 3 to determine whether ACh (via mAChR) and E2 are involved in the consolidation of object representation. Infusions occurring immediately following familiarization influenced novelty preference, consistent with an effect on consolidation. In Chapter 3, a further attempt was made at demonstrating an effect on consolidation, as a delay of 2 hr was introduced between familiarization and infusion on one NOP test trial. Intra-PRh/EC infusions during these trials had no

effect on novelty preference. The enhanced novelty preference following intra-PRh/EC infusions of E2 when infused around the time of familiarization, but not 2 hr later, is consistent with a role in memory consolidation. However, the lack of a delay-dependent effect in Chapter 4 does not provide convincing evidence that these findings reflect an influence on ORM. Taken together, the overall pattern of results taken from the NOP test trials of Chapters 2-4 are consistent with a modulatory effect of ACh (via mAChR) and E2 (via ERβ) novelty preference. However, based on the NOP test alone, is difficult to determine whether these findings reflect a role of ACh and E2 on ORM.

In addition to the NOP test, Chapters 3-4 included the DNMS task. This task does not share the same interpretational challenges, and so conclusions regarding the modulatory effect of E2 on ORM are drawn based on effects observed on this test. In both chapters, divergent effects of E2 were observed on the NOP test and DNMS task. This difference illustrates the challenge with understanding treatment effects on novelty preference. It is hoped that these contrasting results encourage future research to incorporate both tests in the assessment of ORM in rodents.

6.2.5 Other functions of the PRh

In addition to its role in ORM, the PRh is involved in other cognitive abilities, including contextual fear conditioning (Bucci, Saddoris, & Burwell, 2002), and contextual and spatial memory (Burwell, Bucci, Sanborn, & Jutras, 2004; Glenn, Nesbitt, & Mumby, 2003; Ramos, 2013). In addition to cognitive abilities, the PRh is implicated in perception of objects, including the

establishment of object representations. This involves object identification, which is the knowledge that a particular object is one item and that this item is the same across different experiences with the object (Murray & Richmond, 2001). Evidence for a role of the PRh in this ability comes from a study by Buckley & Gaffan (1998). Macaques were tested on a series of visual discrimination problems. Visual discrimination problems involve learning that one image is associated with reward while another is not. These problems require the macaque to distinguish between two images based on visual features. The macaques were presented with images of objects photographed from one point of view. Once these discriminations were learnt, monkeys were then trained to discriminate the objects using different points of view. Learning the new discriminations quicker than the initial set is indicative that features of the objects from the initial point of view were identified in the new set. Macaques with PRh damaged were impaired in the transferring to the new points of view (Buckley & Gaffan, 1998). A subsequent study showed that macaques with PRh lesions are impaired at identifying a different object among an array of different points of view of another object (Buckley & Gaffan, 2000). These two studies demonstrate a role of the PRh in object identification.

The role of the PRh in object perception is thought to be specific to the processing of complex conjunctions of visual features rather than the processing of simple features (Murray & Bussey, 1999). In other words, it is suggested that the PRh is important for discriminating between objects containing a high degree of feature ambiguity. This was examined in a study using the visual

discrimination task. Feature ambiguity was created by creating an image that contains features that are part of both rewarded and unrewarded stimuli. Images of objects that were previously paired with reward or no reward were morphed together. Whether the morphed images contained mostly features of the previously rewarded image or the unrewarded one was manipulated. The low feature ambiguity condition consisted of morphed images containing mostly features of either the rewarded or unrewarded object, whereas the high feature ambiguity condition consisted of morphed images containing close to an equal amount of features from either object. Bussey, Saksida, and Murray (2002) trained macaques on the visual discrimination task using the unaltered images of objects. Once reliable performance was observed, the macaques were then tested on three sets of images, the original pairs, and two pairs of the original images in which the photographs that had been morphed to varying degrees. Macaques with PRh lesions demonstrated impairments with perceptually difficult but not perceptually easy pairs. These impairments were restricted to complex, high-feature ambiguity discriminations, as discriminations between colour and size were not impaired in PRh-lesioned macaques. This study provides convincing evidence that the PRh is involved in visual perception when there is high feature-ambiguity between objects.

Although performance on ORM test requires intact visual perception, the results of the present thesis do not likely to reflect a modulatory effect of ACh and E2 on this ability. Attempts were made to reduce the simple features in common between the objects paired together on the NOP test and DNMS task and so it is

very unlikely that they shared complex features. Therefore, the results described in the present thesis likely reflect and effect of ACh and E2 on ORM.

6.2.6 PRh-mediated effects of ACh and E2 in Alzheimer's disease

Both ACh (Hasselmo, 2006) and E2 (Spencer et al., 2008) modulate HPC functioning and contextual and spatial learning and memory. In addition, E2 mediates ACh release in the HPC by acting on the cholinergic projections originating in the basal forebrain (Spencer et al., 2008; Hammond, Nelson, & Gibbs, 2011). Marriott and Korol (2003) demonstrated increased ACh release in the HPC during a place-learning task mediated by E2. Further, ACh appears to mediate the observed increase in NMDAR binding in the HPC associated with high E2 levels, as well as improve performance on a working-memory task using an 8-arm radial-arm maze (Daniel & Dohanich, 2001). Taken together, these studies suggest an interaction between ACh and E2 on HPC function and spatial learning and memory.

Although not addressed in the present thesis, a similar interaction between ACh and E2 is certainly possible in the PRh. This is corroborated by co-localization of GPER1 on cholinergic neurons in the basal forebrain (Hammond et al., 2011). Damaging cholinergic afferents to the PRh impairs ORM (Winters & Bussey, 2005a; Turchi, Saunders, & Mishkin, 2005). Therefore, one potential mechanism through which E2 may modulate ORM and PRh function may be via these projections.

An interaction between ACh and E2 has important implications for understanding the neurobiology of Alzheimer's disease (AD). Similar to MTL

amnesia discussed in Chapter 1, AD and amnestic mild cognitive impairment are both characterized by deficits in visual recognition memory (Sahakian et al.,, 1988). Women are more likely than men to develop AD (Seeman, 1997), and the severity of their symptoms are typically worse than what is observed in men (Henderson & Buckwalter, 1994). This gender difference appears to be modulated by E as estrogen replacement therapy delays the onset of AD (Pagliani-Hill & Henderson, 1996), and improves memory in patients with AD (Henderson, Pagliani-Hill &, Emanuel, & Dunn, 1994). In addition to E, ACh neurons in the basal forebrain are severely depleted in AD (Auld, Kornecook, Bastianetto, & Quirion, 2002; Craig, Hong, McDonald, 2011). Given that E and ACh are thought to interact to enhance HPC function and spatial learning and memory, Craig and colleagues (2011) have proposed an interaction between these two neuromodulators in AD in women.

MTL structures are severely affected by neurofibrillary tangles that develop in these regions (Gomez-Isla et al., 1996; Mesulam, 2000) and these tangles are thought to contribute to the visual recognition memory impairments in AD (Ally, 2012). Prior to the onset of clinical symptoms, tangles appear in a35 of the PRh (Braak & Braak, 1991) before expanding to the EC, then HPC (Ally, 2012). Despite being the first area affected by AD, and having an important role in ORM, little is known about the contribution of the PRh to AD. Future research examining interactions between neuromodulators like E2 and ACh and how they contribute to PRh function and AD is an important next step to understanding the neurobiology of this debilitating disease.

6.3 Future research considerations

Neuromodulatory mechanisms of ORM are still poorly understood. This is due at least partially to the fact that the majority of available research conducted on mechanisms of ORM focus on the HPC, which is not important in familiarity discrimination. This is particularly problematic for understanding the modulatory effect of E2. One study demonstrated a modulatory effect of E2 replacement in OVX mice on novelty preference and synaptic density in the HPC (Phan et al., 2012). The authors concluded that E2 modulates ORM via increasing spine density in the HPC. However, it is more likely that E2 modulates ORM via actions in the PRh. In the present thesis, higher E2 levels was associated with poorer ORM and reduced density of mature spines in the PRh. Rather than E2 modulating ORM via spine density in the HPC, it may do so via spine density in the PRh. Currently, it is not even known whether the reduced spine density in the PRh following E2 replacement is related to the modulatory effect of E2 on ORM. Future studies should examine whether manipulating spine morphology in the PRh via E2 replacement produces changes in ORM.

One limitation to the present thesis is that modulatory effects were only investigated in the PRh. It is useful to explore whether ACh and E2 influence ORM via actions in other brain structures. Such studies will help determine whether modulatory effects are localized in the PRh. For example, comparisons between the effect of local infusions in the HPC and PRh can help establish dissociations or similarities in the role of ACh and E2 in these two structures.

One important strength to Chapters 3-4 of the present thesis is the inclusion of the DNMS task to measure ORM. This ability is almost exclusively measured in rodents using the NOP test. Although the DNMS task is more time consuming to administer, more definitive conclusions can be made based on treatment effects on this task relative to the NOP test. Including both tests in the present thesis highlights the challenges to understanding the enhanced novelty preference reported in previous studies. It also provides evidence that E2 may have a reverse effect on ORM than previously thought. Incorporating the DNMS task in future investigations is important to further our understanding of ORM, and its neurobiological mechanisms.

Another important consideration involves the possible functional segregation between a35 and a36 of the PRh. Chapter 2 provides evidence that the posterior portion of a36 (and not a35) is involved in novelty-related neuronal activation, and that ACh acts via mAChR to promote this increase. Chapter 5 shows that high E2 levels are associated with reduced mature spine density in a35 (and not a36). While a36 is implicated in one study, a35 is implicated in the other. The present thesis does not provide any insight into the potential functional segregation between these two sub-regions. However, it does suggest that ACh and E2 modulate ORM via different sub-regions of the PRh. Future studies are needed to determine the role of these two sub-regions in ORM before conclusions can be made about why the disparity was observed.

One final consideration relates to whether E2 enhances or impairs ORM. Although the results from the DNMS task in Chapters 3-4 suggest the latter, both

the dose and duration of E2 treatment are known to influence the direction of the effect on cognition (Korol, 2004; Zurkovsky et al., 2007). Future studies manipulating these factors are needed before conclusions can be made about how E2 modulates ORM.

6.4 Conclusions

The results from the present thesis provide convincing evidence that ACh and E2 modulate performance on tests of ORM. When using the NOP test alone, they suggest that mAChR and ERβ are both associated with enhanced novelty preference. Novelty preference is modulated when infusions occur immediately before (Chapters 2-4), immediately after (Chapter 2-3), but not 2 hr following familiarization (Chapter 3), suggesting that ACh (via mAChR) and E2 (via ERβ) enhance novelty preference by acting both during familiarization and the consolidation of object representations.

Although consistent with enhancing ORM, the failure to observe delay-dependent effects on the NOP test (in Chapters 2 and 4) are also consistent with an enhancement of non-cognitive processes that influence novelty preference. This possibility is supported by the impairments observed on the DNMS task in Chapters 3-4 following intra-PRh infusions of E2. Performance on the DNMS task following mAChR antagonism was not addressed in the present thesis. Since previous research demonstrates that mAChR influences DNMS task performance in human and non-human primates, it is entirely possible the observed effects in Chapter 2 reflect a modulatory effect of ACh (via this receptor) on ORM. However, the divergent results on the NOP test and DNMS task in Chapters 3-4, including an inconsistent effect of a selective ERβ agonist

on the two tests suggests that E2 does not enhance ORM, but instead, impairs this ability.

In addition to modulating performance on tests of ORM, ACh and E2 appear to influence PRh function. Neuronal activation (as measured via Fos-IR) is enhanced in the posterior a36 following novel- relative to familiar-object exploration, and this difference is attenuated by a mAChR antagonist. PRh activation following stimulus presentation has previously been proposed to reflect a neuronal mechanism of familiarity discrimination, and so the results of the present thesis suggest that ACh may modulate this mechanism via mAChR.

High E2 levels are associated with reduced density of mature spines in a35, but not a36 in the PRh, suggesting it may reduce excitatory synaptic plasticity in this region. This finding is particularly important as it provides a potential mechanism through which E2 may modulate ORM.

The present thesis provides new insights into modulatory mechanisms of ORM in the PRh. The findings indicate that ACh and E2 influence performance on tests of ORM by acting within the PRh, a structure previously overlooked by many. Comparisons between the NOP test and DNMS task also provide new insight into challenges with using the NOP test as a sole measure of ORM.

This thesis also demonstrates that PRh-mediated neural correlates of learning and memory are influenced by both ACh and E2. The studies described above provide support for the idea that ACh and E2 modulate ORM via novelty-related neuronal activation and synaptic density in the PRh. These results provide important insight into potential ways ACh and E2 might influence

cognitive decline associated with AD. In particular, determining the unique and combined actions of ACh and E2 within the PRh is an important next step in understanding the development of recognition memory impairments associated with AD.

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