The Effects of Ovarian Hormones on Memory Bias and Progesterone Receptors in Female Rats

by

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ABSTRACT

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Ovarian hormones can bias female rats to use one memory system over another when navigating a novel or familiar environment, resulting in a memory bias. High levels of estrogen (E) promotes places memory while low level of E promotes response memory. However, little is known about the effects of progesterone (P) on memory bias. Experiment 1 determined whether P affects memory bias. Ovariectomized (OVX) female rats were trained in a plus-shaped maze, which assesses memory system bias, and received one of three hormonal treatments: Low 17β estradiol (E2), high E2 or high E2 + P. P did affect memory bias by reversing the effects of high E2 when rats receive P one hour prior to testing. To understand the mechanisms by which P affect memory bias in the hippocampus (HPC), antibodies directed at nPR, mPRβ and mPRδ were examined. The effects of low E2, high E2, and high E2+P were examined on immunoreactivity to these receptors in the HPC. All three receptor-types were found in the female rat HPC and were found to be insensitive to hormone administration. The presence of these receptors suggests that P can exert both genomic and non-genomic effects in the HPC. Other brain areas involved in memory bias remain to be examined further.

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Abbreviations

ALLO	Allopregnanolone
BSA	Bovine serum albumin
CA1-3	*Cornu Ammonis* layers 1, 2, and 3 of the hippocampus
CNS	Central nervous system
DG	Dentate gyrus region of the hippocampus
dSTR	Dorsal striatum
E	Estrogens
E2	17β-estradiol
EB	Estradiol benzoate
EC	Entorhinal cortex
ER	Estrogen receptors
GABA	λ-aminobutyric acid
GPER1	G-protein-coupled estrogen receptor 1
HPC	Hippocampus
IR	Immunoreactivity
IP	Intraperitoneal
mER	Membrane-associated estrogen receptors
mPFC	Medial prefrontal cortex
mPR	Membrane progesterone receptors
NDS	Normal donkey serum
nPR	Nuclear progesterone receptors
OR	Odds ratio
OVX	Ovariectomy
P	Progesterone
PAQR	Progestin and adipoQ receptor
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PGRMC1	Progesterone receptor membrane component 1
PR	Progesterone receptor
RT	Room temperature
SLM	Stratum lacunosum-moleculare
TBI	Traumatic brain injury
TBS	Tris-buffered saline
Tx	Triton-X

Figures and tables

The ovaries primarily secrete estrogens (E) and progesterone (P). Traditionally, the primary functions of these ovarian hormones were thought to be only to regulate the female reproductive system, maintain pregnancy, and promote lactation. In naturally cycling females, ovarian hormone levels fluctuate across the menstrual cycle in women, which is called the estrous cycle in female rats. In addition to being important for reproductive and maternal behaviors, we now know that they have much more widespread effects in regions of the brain involved in cognition. Specifically, human and rodent research suggests that hormonal levels can account for major sex differences in the ability to navigate an environment (Brake & Lacasse, 2018). The effects of E and its receptors on spatial navigation have been studied extensively over the past few decades (for review, see; Hussain et al., 2014), yet less is known about the effects of P on spatial navigation despite that it co-fluctuates with P. Moreover, the distribution and function of progesterone receptors (PRs) in areas of the female brain outside the hypothalamus are virtually unknown.

Multiple memory systems

When navigating an environment, rats use one or more memory systems to reach a goal. Tolman and colleagues (1946) found that rats navigate through an elevated plus maze to find food by using different learning strategies. These types of memories are referred to as place (or spatial) memory and response (or habitual motor) memory. Response memory refers to an egocentric strategy that relies on internal cues by the body, such as habitual turns, to navigate an environment (Hussain et al., 2014). Place memory refers to an allocentric strategy that relies on multiple landmarks from the environment and creates a cognitive map to assist navigation (Hussain et al., 2014). These two types of memory rely on different regions of the

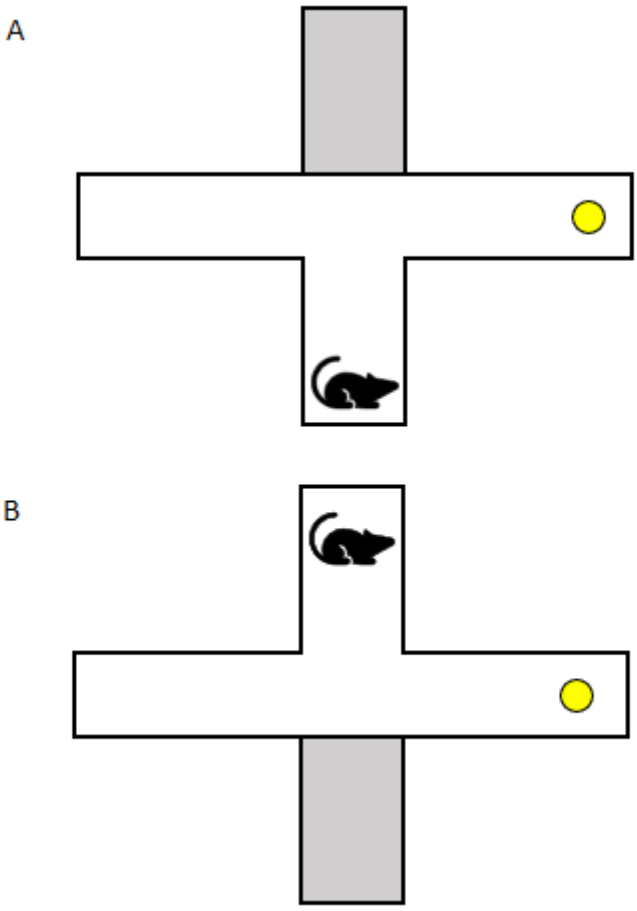


Figure 1. An example of a modified plus maze. (A), A rat is trained to find a reward in a baited arm. (B) Upon reaching criterion, the rat placed 180 degrees to the start arm. If the rat turns right, it was scored as using response memory and if it goes to the same spatial location, it was scored as using place memory.

brain. Impaired place memory has been observed in rats with hippocampal lesions (McDonald & White, 1994; Packard & McGaugh, 1996). On the other hand, damage to the dorsal striatum (dSTR) was found to impair response memory (Featherstone & McDonald, 2004), suggesting that place memory is mediated by the hippocampus (HPC) and response memory is mediated by the dSTR. Under normal circumstances, these two memory systems are thought to interact. However, when rats are put in a plus maze (Figure 1) where either memory system could be used, a bias of using one memory system over the other can emerge. In early maze trials, male rats will predominantly use place memory, but with extended training, they will shift to response memory (Packard & McGaugh, 1996; Chang & Gold, 2003). While it is thought that rats use both of these two memory systems in navigation, some evidence suggests that they may be competitive. When the male rat HPC was lesioned, place memory was impaired while striatal-mediated response memory was enhanced (White & McDonald, 2002). When rats had damage to the dSTR, response memory was impaired, but place memory was enhanced (White & McDonald, 1995). White and McDonald (1995) concluded that there appear to be at least two competing memory systems in the brain such that when one is impaired, the other is enhanced.

Ovarian hormones and memory systems bias

There is evidence that memory system use can have an inherent bias based on the hormonal profile in female rats. Korol and colleagues (2004) conducted a study to determine whether hormonal fluctuations across the estrous cycle would have an impact on memory bias in the plus maze. They found that rats were more likely to use place memory in the proestrus phase, when both 17β-estradiol (E2), the most potent form of E, and P levels were at their

peak. In the estrus phase, characterized by low levels of E2 and P, rats were more likely to use response memory. Several studies since on ovariectomized (OVXed) rats with E2 replacement have been conducted. These E2 replacement levels were meant to reflect the levels of E2 in proestrus and estrus respectively. Rats that received chronic low, along with pulsatile high, E2 replacement predominantly used place memory and rats that received chronic low E2 predominantly used response memory (Korol & Kolo, 2002; Korol et al., 2004; Hussain et al., 2013; Almey et al., 2014; Quinlan et al., 2008, 2013). Therefore, systemic administration of high E2 has been associated with a bias towards place memory and low levels of E2 have been associated with a bias towards response memory.

Studies using central infusions of E2 into the dSTR and dorsal HPC have extended our understanding of the relationship between E2 and the brain areas involved in memory bias. After having E2 infused directly into the dSTR prior to training, female rats performed more poorly on a response learning task (Zurkovsky et al., 2011). Conversely, after E2 was injected into the dorsal HPC, female rats showed an improvement in place learning (Zurkovsky et al., 2007). These results suggest that high levels of E2 in the dorsal HPC are associated with improved place learning, while lower levels of hippocampal E2 are associated with improved response learning. During the proestrus phase when E2 levels are higher, an increase in dendritic spine density in CA1 pyramidal neurons of the HPC was observed. In addition, high E2 replacement in OVX rats increased HPC dendritic spine density (Gould et al., 1990; Woolley et al., 1990) as well as HPC synaptic proteins (Brake et al., 2001), suggesting that high E2 increases synapses in the dorsal HPC. In addition, E2 has shown to increase neuron excitability in the HPC (Wooley et al., 1990).

The medial prefrontal cortex (mPFC) also plays a role in memory bias. The mPFC has reciprocal projections to the HPC and dSTR (Jay & Witter, 1991; McDonald & White, 2002) and has been shown to be involved in memory system bias. Inactivation of the prelimbic and infralimbic regions of the mPFC impaired male rats from switching from one memory system to another (Ragozzino et al., 1999; Rich & Shapiro 2007). E2 has been shown to increase the number of dendritic spines in the PFC compared to OVX rats (Khan et al., 2013) and 15 min after receiving infusions of E2 into the mPFC, female rats were more likely to use place memory in the ambiguous T-maze, whereas the sham infusion rats were more likely to use response memory (Almey et al., 2014).

Estrogen receptors in the PFC, dSTR and HPC

Three types of estrogen receptors (ERs) have been discovered so far; ERα and ERβ are nuclear steroid receptors that form a homo- (ERα-ERα or ERβ-ERβ) or hetero- (ERα-ERβ) dimer in the cytoplasm and translocate to the nucleus to exert genomic effects. Recently, ERα and ERβ have also been identified at the cell membrane and when located there, they are referred to as membrane-associated receptors (mERs; Almey et al., 2015). These mERs are thought to exert rapid non-genomic effects, such as altering membrane permeability and second messenger cascades (Almey et al., 2015). In addition, there is a third ER that is observed on the cell membrane that was formerly known as the orphan G-protein coupled receptor 30 that is now identified as G-protein coupled ER1 (GPER1). GPER1 likely acts through second messenger cascades resulting in rapid non-genomic effects or delayed long lasting genomic effects. Specifically, GPER1 activation can involve Gs proteins which increase adenylate cyclase activity

(Alexander et al., 2017). GPER1 has shown to activate an inhibitory Gi/o protein, ultimately activating phosphoinositide 3-kinase within the cell (Alexander et al., 2017).

All three ERs had been shown to be exclusively located at extranuclear sites in the mPFC, dSTR and HPC using electron microscopy (Almey et al., 2015). In the mPFC, most of these ERs are localized in axons and axon terminals; GPER1 was the most abundant ER at the axon terminals and ERβ was the most abundant on axons (Almey et al., 2014). In the dSTR, ERs are mostly found in axons and glial profiles, less so in dendrites and axon terminals (Almey et al., 2012); 49% of all mERβs, 35% of all mERαs and 36.4% of all GPER1s were all localized in axons (Almey et al., 2012)**.** In addition, dual labeling revealed that mERα and GPER1 are located on GABA (gamma aminobutyric acid) and acetylcholine neurons in the dSTR (Almey et al., 2016; Almey et al., 2012). Studies using light microscopy confirmed moderate levels of nuclear ERα and ERβ and relatively higher levels of GPER1 in the HPC (Mitra et al., 2003; Zhang et al., 2002; Hazell et al., 2009). In addition, an electron microscopy study showed that mERα was localized in the various layers of the *Cornu Ammonis* 1 (CA1) and dentate gyrus (DG; Milner et al., 2001). Within these regions of the HPC, 50% of mERα was observed on axons and axon terminals and 25% of mERα was observed on dendritic spines (Milner et al., 2001). Some mERα has been observed on cholinergic presynaptic terminals (Towart et al., 2003) and others have been associated with synaptic vesicles in a subset of GABAergic presynaptic profiles in the CA1 region of the HPC (Hart et al., 2007). Milner and colleagues (2005) also localized mERβ on extranuclear sites in the CA1, CA3, and DG of the HPC. Approximately 40% of mERβ observed on postsynaptic sites (dendritic spines and dendritic shafts) and about 30% of mERβ was observed on presynaptic sites (Milner et al., 2005). Lastly, GPER1 has been observed at the plasma

membrane on the pyramidal neurons of the CA2 region at pre- and post-synaptic sites (Funakoshi et al., 2006) and on dendritic spines in the CA1 region of the HPC (Akama et al., 2013). In addition, they have also been found on interneurons throughout the HPC.

GABA neurons are involved in regulating E2-induced spine density in the HPC (for review, see; Cooke & Wooley, 2004). Increases in spine density *in vitro* is preceded by a decrease in GABAergic input to spiny neurons (Murphy et al., 1998). E2 administration *in vivo* disinhibits CA1 pyramidal cells within 24 hours, preceding the increase in spine density (Rudick & Wooley, 2001). Therefore, E2 and potentially its receptors may be needed for the disinhibition of GABAergic activity in order to increase spine density in the HPC.

Furthermore, E2-induced changes in dendritic spine density in the HPC has shown to influence behaviors associated with the HPC. Sandstorm & Williams (2001) found a correlation between spatial working memory and E2-induced increases in dendritic spine density: Spatial working memory was improved in the Morris water maze when rats were tested 1-4 days post-E2 administration, when spine density was elevated. No improvement in spatial working memory was observed when spine density levels were low on day 0-1 and day 9-10 (Sandstorm & Williams, 2001).

Together, these results suggest that E2 may play a role in neuronal transmission in the mPFC, dSTR and HPC. Most ERs in the mPFC are localized on axons and axon terminals, thus E2 may alter presynaptic transmission in this region. However, it has yet to be determined which neurons these ERs are associated with. ERs in the dSTR are also mainly found in axons, therefore may have a role in presynaptic transmission, specifically on cholinergic and GABAergic neurons. Similar to the dSTR, mERα may be implicated in hippocampal cholinergic and

GABAergic presynaptic transmission. Moreover, mERβ's presence on axons and dendrites in the HPC may be implicated in both pre- and post-synaptic transmission. Finally, the presence of all three ERs on dendritic spines in the HPC may be one way that E2 increases dendritic spine densities, synaptic proteins, and neuronal excitability in the CA1 region of HPC. These events may be one mechanism by which estrogens increase the use of spatial memory in female rats.

Progesterone and cognition

Though P fluctuates in conjunction with E, less is known about the effects of P on cognition. Studies have shown that P can have disruptive effects on memory performance while others have shown beneficial effects (Barros et al., 2015). For instance, Warren & Juraska (1997) showed that females in the proestrus phase, when both E2 and P are high, were less efficient in the Morris water maze, a measure of spatial memory performance, than females in the estrus phase (low E2 and P). Additionally, when OVX rats were trained in the Morris water maze, administration of E2 and P prior to testing resulted in a longer latency to complete the task and a longer path length (Chesler & Juraska 2000). This impairment was not seen when E2 or P alone was administered, suggesting that the disruptive effects of P are dependent on the presence of E2 and vice versa. Such effects of P may also be dose dependent. When OVX female mice were injected with E2 plus 20mg/kg of P, they displayed a longer swim distance (i.e., poorer performance) in the eight arm spatial water maze task compared to 10mg/kg of P (Harburger et al., 2007).

In contrast, other studies have shown that P enhances memory performance. In this case, the timing of P administration is also important. Frye *et al*. (2009) showed that OVX rats performed better in the water maze and in the Y-maze object recognition task when P was

administered instantly after training. Moreover, infusing P into the dorsal HPC enhances object recognition in female OVX mice when P was given immediately after training, but not when P was administered two hours post-training (Orr et al., 2009). Thus, it has been hypothesized that P facilitates memory consolidation (Barros et al., 2015). In sum, P can have either disruptive or beneficial effects on memory; these effects may be dependent on the dose and on timing of P administration.

P may also play a role in neuronal transmission, particularly in the HPC. As mentioned earlier, dendritic spine density has been found to naturally fluctuate during the estrous cycle in female rats (Woolley et al., 1990). Apical dendritic spines in CA1 pyramidal neurons of the HPC were found to be significantly lower in females in the estrus phase (low E2 and P) than rats in the proestrus phase (high E2 and P). These effects were not observed in the CA3 and DG of the HPC. In OVX female rats, dendritic spine density in the CA1 region of the HPC drastically decreases, but with E2 replacement, this effect is prevented. Within five hours of P administration to E2 replacement rats, apical and basal dendritic spine density increased compared to E2 replacement alone. Interestingly, Woolley and McEwen (1993) found that P has biphasic effects on apical dendritic spine density in CA1 pyramidal neurons. P administration increases dendritic spine density in the first 2-6hours, after which there is a sharp decrease in spine density. By the 18th hour, dendritic spine density goes down to values equivalent to a rat that had undergone OVX 6 days ago (Woolley & McEwen, 1993). Additionally, the administration of a progesterone receptor antagonist (RU 486) in intact female rats in the proestrus phase resulted in an inhibition of the decrease in spine density that occurs in the transition from proestrus to estrus (Woolley & McEwen, 1993). In sum, short term (5 hours) P

administration increases apical dendritic spine density in CA1 pyramidal neurons, while long-term P decreases in spine density.

Progesterone receptors

P classically acts through nuclear PRs (nPRs). These were first characterized in the 1970s (Milgrom & Baulieu, 1970) and have been localized throughout the brain. So far, there are two isoforms of nPRs that have been discovered: PR-B (120 kDa) and the N-terminally truncated form, PR-A (86 kDa), both of which are derived from the same gene. Once bound to P, nPRs form a homodimer in the cytoplasm and translocate to the nucleus where they interact with progesterone response elements (PREs) in DNA, ultimately modifying gene transcription. This effect occurs on the timescale of hours (Wilkenfeld et al., 2018). PR-A and PR-B regulate gene transcription differently. In the uterine epithelium of mice, PR-B functions as a transcriptional activator while PR-A functions as a transcription repressor on PR-B (Conneely et al., 2002).

Early studies found that E2 induces nPRs in the uteri of rats and mice (Milogrom et al., 1970). There are two distinct nPR systems in the brain: E2-inducible PRs and non-inducible PRs (MacLusky & McEwen 1980). E2-inducible and non-inducible nPRs are structurally indistinguishable from each other (MacLusky & McEwen 1980). OVX female rats have low nPR in the hypothalamus and preoptic area, whereas E2 replacement significantly increases these levels (MacLusky & McEwen 1978). Conversely, E2 administration has no effect on nPRs in the midbrain and cerebral cortex (MacLusky & McEwen 1978). Parsons *et al*. (1982) found that E2-inducible nPRs in the CA1 subfield of the HPC. nPR expression does not change in the HPC over time in naturally cycling rats (Guerra-Araiza, Cerbón, et al., 2000; Guerra-Araiza et al., 2003). However, when OVX female rats receive E2 replacement, hippocampal nPR levels increase

(Guerra-Araiza et al., 2003). The addition of P to the E2 treatment reduces nPRs (Guerra-Araiza et al., 2003). In sum, P downregulates E2-sensitive nPRs. Those that are insensitive to E2 are also insensitive to P. Whether nPRs are present in the dSTR and mPFC and whether they are inducible by E2 has not been examined.

P can also act via membrane PRs (mPRs). mPRs were initially thought to be a type of G-protein coupled receptor (GPCR) because they were found to mediate rapid intracellular signalling cascades through G-protein activation and have structural resemblances to a GPCR. By definition, GPCRs are structurally characterized by the presence of seven transmembrane domains with an extracellular N-terminus and an intracellular C-terminus (Tang et al., 2006). However, due to the lack of significant sequence similarities to major GPCRs (e.g., metabotropic neurotransmitter receptors or adrenergic receptors) and their N-terminus being present inside the cell instead of out, mPRs were reclassified as members of the class II progestin and adipoQ receptor (PAQR) family (Tang et al., 2006). Of the three classes of PAQR, only class II responds to P and only this class couples to G-proteins (Smith et al.,2008). Up to now, five mPRs have been discovered. The first of them, mPRα (PAQR7), was discovered from spotted seatrout ovaries (Zhu et al., 2003b). mPRβ (PAQR8) and mPRγ (PAQR5) were subsequently identified in humans and other vertebrates (Zhu et al. 2003a). These three mPRs inhibit adenylyl cyclase, suggesting that they activate an inhibitory Gi protein (Pang et al., 2013). While mPRβ is highly expressed in neural tissue, mPRα and mPRγ are highly expressed in the rat ovaries and fallopian tubes suggesting their primary function is to regulate reproductive functions (Zuloaga et al., 2012). The last two uncharacterized members of the PAQR family (mPRδ [PAQR6] and mPRε [PAQR9]) were later identified by expressing human complementary DNA (cDNA) in yeast cells

(Smith et al., 2008). These relatively novel mPRs activate a stimulatory Gs protein (Pang et al., 2013). mPRs can exert rapid effects taking place in seconds to minutes (Gellersen et al., 2008), or longer lasting genomic effects, indirectly altering gene expression, via second messenger cascades or various signal transduction pathways.

Pang and colleagues (2013) mapped the expression of mRNA levels for each mPR subtype in the human brain. In the HPC, mPRδ and mPRβ are the most abundant followed by mPRε, mPRα and mPRγ, respectively (Pang et al., 2013). In OVX female rats with estradiol benzoate (another potent E) replacement, mPRβ levels are moderate to high in CA2 and CA3 regions of the HPC and low in the CA1 and DG (Zuloaga et al., 2012). In mRNA studies, mPRα is almost undetectable and mPRβ has moderate levels of expression in the HPC (Intlekofer & Petersen, 2011b). Additionally, E2 and P does not regulate mPRα mRNA levels in any brain regions in female rats, while mPRβ mRNA levels increase with E2 administration, decrease with P administration and increases with E2 plus P administration in the hypothalamus (Intlekofer & Petersen, 2011a). Thus, mPRβ seems to display E2-inducible properties, while mPRα does not. Whether the other mPR subtypes are inducible by E2 or present in the HPC, dSTR, or mPFC in the female rat has yet to be examined.

Rationale

Rats navigate a modified plus maze using both place and response memory. Depending on ovarian hormone levels, female rats will have a bias towards one memory system over the other. High E2 biases rats towards place memory, while low E2 biases rats towards response memory. While the effects of P on place memory has been examined, its effects on memory

bias has not. Thus, in Study 1, the role of P in memory system bias was examined. OVX female rats were trained in a modified plus maze and received one of three hormonal treatments: Low E2, high E2 or high E2 + P. P has the potential to exert both genomic and non-genomic effects via mPRs, but only genomic effects through nPRs. As such, in Study 1, the high E2 + P group was subdivided into three groups based on the timing of P injection (15 minutes, 1 hour or 4 hours) prior to the probe test to determine if P exerts genomic or non-genomic effects on memory bias. It was hypothesized that low E2 rats would be more likely to use response memory and high E2 rats would be more likely to use place memory. Studies have shown that P, when administered prior to testing, impairs spatial memory performance (Warren & Juraska, 1997; Chesler & Juraska, 2000). The impairment effects were observed when P was injected 4 hours prior to testing. Four hours is sufficient for genomic effects to occur, while one hour and 15 minutes are not. Johansson *et al.* (2002), observed spatial impairments as early as 8 minutes prior to testing when rats were injected with a derivative of P, allopregnanolone (ALLO). Therefore, to investigate the possibility of P exerting non-genomic effects, rats were injected one hour or 15 minutes prior to testing. It was hypothesized that rats treated with E2+ P would be more likely to use response memory.

Certain PRs are upregulated by E2 (E2-sensitive), while other PRs are E2-insensitive. These PR properties are region specific within the CNS. Despite P's effects on female cognition, the mechanisms by which P acts on its receptors and the distribution of PRs, ultimately affecting memory, has yet to be determined. Therefore, in Study 2, the HPC was examined for the presence of PRs. The distribution of nPRs and the two most abundant mPRs expressed in the human and/or rodent brain, mPRδ and mPRβ, were localized. Because some PRs are

inducible by E2 in certain brain areas, it was also determined whether PRs were E2-sensitive. OVX female rats were treated with low E2, high E2 or high E2+P and subsequently immunolabeled for mPRβ, mPRδ and nPRs. A western blot analysis was performed to confirm the specificity of all primary antibodies used in the study. Additionally, it is well established that the hypothalamus contains nPRs. Thus, the hypothalamus was also analysed, (i.e; the arcuate nucleus [ARC] and the ventrolateral part of the ventromedial hypothalamus [VMHvl]) as a positive control to test the sensitivity for the antibody used. It was hypothesized that nPRs and mPRβ would be present in the HPC and nPRs found in the CA1 region of the HPC to be E2-inducible and downregulated with P treatment. PRs that are not affected by E2 treatment were not expected to be affected by P treatment.

Methods

Experiment 1: Progesterone and memory systems bias

Subjects

This experiment used 120 female Long Evans rats that weighed 220-240g on arrival. 89 of these rats arrived from Charles River, St-Hyacinthe, QC and 31 were acquired through internal breeding from the Animal Care Facility at Concordia University. Rats were housed in pairs in shoe-box cages (25.5 cm wide×46.6 cm long×21.6 cm high) and handled for five days to familiarize them with the researchers. Animals were housed in a reverse 12-hour light-dark cycle (1900h to 0700). Standard rat chow and water was available *ad libitum* prior to behavioral training and surgeries. Animals were habituated in the modified plus maze daily for a period of two weeka prior to surgery. All procedures adhered to guidelines set forth by the Canadian

Council on Animal Care and were approved by the Concordia Animal Research Ethics Committee.

Surgery and Hormone Replacement

Ovariectomy. Rats were anesthetized with isofluorine and oxygenated at a ratio of 1:200. Ovaries were removed bilaterally through a dorsal incision using standard aseptic procedures. The incision was sutured using 9 mm stainless steel surgical staples (EZ clips; Stoelting Co., Wood Dale, Illinois). Post-surgery, rats received the analgesic Anafen (0.1mL/animal, s.c.) and the antibiotic penicillin G (0.1mL/animal, s.c.). Approximately five days of recovery was allowed and 0.5oz hydrogel (ClearH_20, Portland, ME) was given daily to maintain hydration. Five days following surgery, 10 choice trial training began.

Hormone replacement. To maintain a baseline level of circulating E2, capsules made of Silastic tubing (1cm long; Dow Corning), containing 5% β-Estradiol (Sigma, Ontario) in cholesterol (Sigma) were implanted subcutaneously lateral to the incision site after the ovariectomy. Rats were trained and tested within a 3-week time frame, when plasma concentrations of E2 has been found to mimic the low E2 levels in the diestrus phase of the estrous cycle (Almeyet al., 2013; Overpeck et al., 1978).

Rats were randomly assigned to one of three hormone treatment groups (n=24/group): low E2, high E2, high E2 and P. The low E2 group was injected with sesame oil (0.1ml, s.c.) 24 hours and 4 hours prior to behavioural testing. The high E2 group was injected with E2 (10µg/kg, s.c; Sigma) 24 hours and injected with sesame oil (0.1ml, s.c.) 4 hours prior to behavioural testing. The high E2 and P groups were injected with E2 (10µg/kg, s.c; Sigma) 24

hours prior behavioural testing and injected with P (500µg/kg, s.c.), 15 minutes, 1 hour or 4 hours prior to behavioral testing.

Modified plus maze

All habituation, training and testing were conducted in a modified plus maze. The plus maze was in a dimly lit room with overhead red fluorescent lighting, and a lamp facing the ceiling (40 W light bulb). It was composed of four arms (75cm long each) with dark grey walls (28cm high), clear Plexiglass ceiling panels, and a grid flooring on a table (1m high). The plus maze was divided into the following chambers by black sliding doors: the start chamber, the start arm, two choice chambers (i.e. the goal arms that contained the food reward), and the probe arm. The doors were manually operated by a pulley system from a marked location in the room. For habituation and behavioral training, the probe arm was blocked off and the start arm, which was 180 degrees relative to the probe arm, was opened (Figure 1A). Conversely, during the probe trial, the start arm was blocked off and the probe arm was opened (Figure 1B). Other spatial cues which included cupboards, posters on the walls and the researcher standing in a designated zone, operating the sliding doors remained in the same spatial location.

Habituation, Training and Food Restriction

After handling the rats for 5 days, rats were given 10 minutes to explore the T-maze with the probe arm blocked for the next 3 days. On day 9, habituation time was then reduced to 5 minutes. Froot Loops (Kellogg's^{TM}), the food reward, were placed at the end of either goal arms in small stainless steel bowls. On day 14, rats were randomly assigned to be trained to a target goal arm (left or right) containing the food reward. Froot Loop crumbs were scattered

under the grid flooring throughout the maze to mask odor cues. Additional amounts of Froot Loops were placed under the grid flooring at the end of each goal arm to exhibit the presence of the reward at the end of either goal arms. On day 19, rats were OVXed (Figure 2).

After a five day recovery period, rats proceeded to the 10 choice trial training on day 25. To further motivate the rats to retrieve their reward, they were food restricted, therefore single housed from then on. Their weight was maintained at 80% of their free-feeding levels. The 10 choice trials consisted of the rat going to one of the goal arms to retrieve the food reward. Once the rat had fully entered either goal arm, the sliding door would close behind it. If the rat entered the randomly assigned goal arm, it would be able to acquire the food reward. If the rat entered the incorrect goal arm, it was given 10-30 seconds to notice that there was no reward at the end of the goal arm before being removed from the maze. Rats were considered to have met criterion to test if they achieved 8 of 10 correct trials for three consecutive days. Once they have met the criterion to test, rats were tested the following day. Based on previous findings, rats take up to 15 to 20 days to reach criterion. Rats that did not reach criterion within 20 days of the 10 choice trial training were excluded from the study and additional rats were tested to compensate for the reduced *n*.

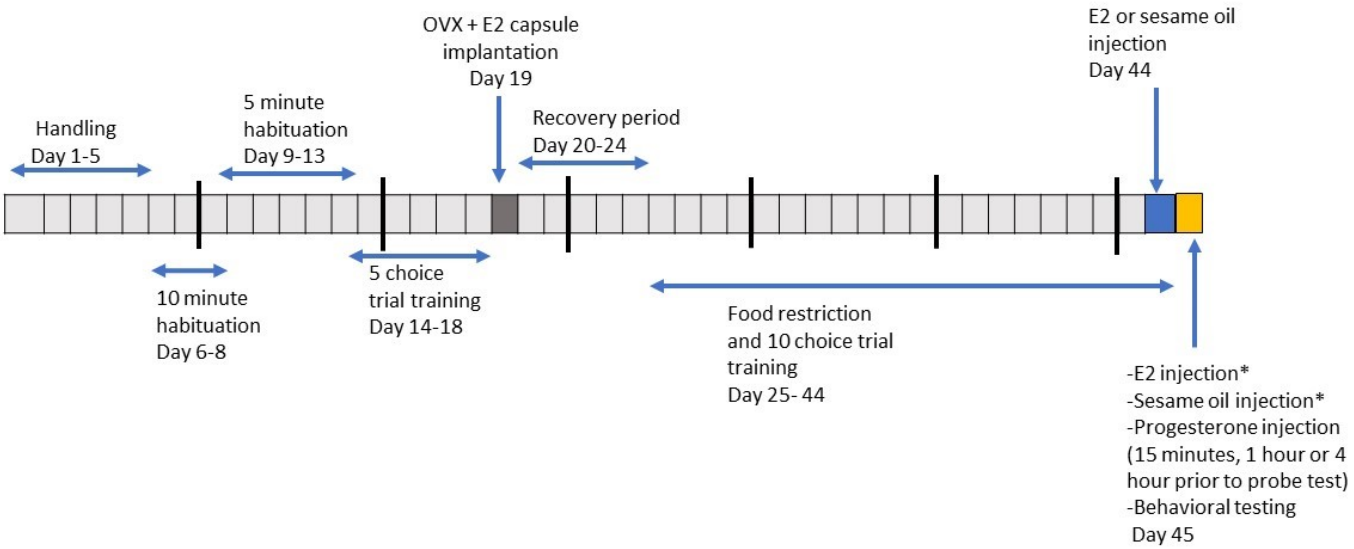


Figure 2. Experimental timeline for all rats. Each box represents one day. Asterisks indicate that injection was administered 4 hours prior to behavioural testing. OVX., ovariectomy.

Behavioral Testing

On testing day, rats underwent the same 10 choice trials as training. Once the 10 trials were completed, the start arm was blocked off and the probe arm, 180° from the start arm, was opened. The rat was placed in the probe arm and underwent a probe trial. The food reward was placed at the end of both goal arms. If the rat turned into the goal arm that had been baited during the training trials (i.e. the same spatial location), it was deemed that the rat was using place memory. If the rat turned in the opposite direction, it was deemed that the rat was using response memory.

Statistical Analysis

This experiment was a between-subjects design with categorical data and thus, nonparametric statistics were used. A Chi-square (χ^2) analysis was conducted within each hormone condition to determine whether there was a significant difference in memory system use in the probe trial. In addition, based on a priori predictions, a Chi-square analysis was also conducted between the high E2 group against all high E2+P groups to see if progesterone had any significant effect on memory bias. Finally, an odds ratio (OR) for each of these analyses was also calculated in lieu of effect sizes.

Experiment 2: PR distribution in the HPC

Subjects

This experiment used 18 female Long Evans rats from Charles River, St-Hyacinthe, QC that weighed 220-240g on arrival. Rats were housed in pairs in shoe-box cages (25.5 cm wide×46.6 cm long×21.6 cm high). The rats were handled daily from time of arrival until

completion of the experiment except for during recovery days post-surgery. The colony room used a reverse 12-hour light-dark cycle (1900h to 0700). Standard rat chow and water was available *ad libitum* throughout the experiment. All procedures adhered to guidelines set forth by the Canadian Council on Animal Care and were approved by the Concordia Animal Research Ethics Committee.

Ovariectomy. Surgeries were conducted as described previously in experiment 1. All rats received one week to recover prior to being handled again.

Hormone replacement

To maintain a baseline level of circulating E2, an E2 capsule as described previously in experiment 1, was implanted subcutaneously lateral to the incision site after the ovariectomy.

Rats were randomly assigned into one of three groups: low E2 (*n*=6), high E2 (*n*=6), high E2 and P (*n*=6). Two weeks following surgeries, the low E2 group was injected with sesame oil (0.1ml, s.c.) 48 hours and 24 hours before perfusions. The high E2 group was injected with E2 (10µg/kg, s.c; Sigma) 48 hours prior and injected with sesame oil (0.1ml, s.c.) 24 hours prior perfusions. The high E2 and P group was injected with E2 (10µg/kg, s.c; Sigma) 48 hours prior and injected with P (500µg/kg, s.c.), 24 hours prior to perfusions.

Tissue preparation

Rats were injected with sodium pentobarbital (150 mg/kg, i.p.) and then transcardially perfused through the ascending aorta with 250mL of 0.1M phosphate buffer (PB) and subsequently with 250mL of fresh 4% paraformaldehyde (PFA) in PB (pH7.2). Brains were removed and transferred into a 4% PFA in PB solution for 48hours and then transferred into a 30% sucrose solution for 48-72hours at 4°C. Based on the rat brain atlas by Paxinos and Watson

(1998), the PFC (Bregma: 5.16-2.52), the dSTR (2.28 to -0.60) and the dorsal HPC (-2.28, -4.36) were sectioned at 30µm thickness using a Shandon Cryotome FE and FSE (Thermo Fisher Scientific; Waltham, MA). All rat brain slices were stored at -20°C in a 2mL microcentrifuge tube containing cryoprotectant (30% sucrose and 30% ethylene glycol in PB). There were 6-10 brain slices in each microcentrifuge tube that represented the entirety of each of the brain regions of interest.

Immunohistochemistry

Free floating tissue sections were rinsed in 50mM phosphate buffered saline (PBS) for 10 minutes and then in PBS with 0.3% Triton-X (Tx) for 30 minutes. Tissues were then incubated in blocking solution composed of 6% normal donkey serum (NDS), 3% non-fat dry milk and 0.3% bovine serum albumin (BSA) in PBS-Tx for 1 hour at room temperature (RT). Tissue was next incubated in primary antibody solution composed of 2% NDS, 3% non-fat dry milk, 0.3% BSA and the primary antibody for 48hours at 4°C. One of three primary antibodies used were for each set of sections. Tissue was incubated with rabbit antibodies against either mPRβ (1:1000; PA3-881, ThermoFisher Scientific), mPRδ (1:500; NBP1-59477, Novus Biologicals, Oakville, ON) or nPR (1:100; MA5-14505, ThermoFisher Scientific). Tissue was subsequently rinsed for 30 minutes with PBS-Tx and incubated with Alexa Fluor 647 AffiniPure donkey anti-rabbit IgG secondary antibody (1:1000; Jackson ImmunoResearch, West Grove, PA) for one hour at RT. Tissue was subsequently rinsed in PBS-Tx for 30minutes and PBS for 10 minutes. Sections were mounted on gelatin coated microscope slides (0.5% gelatin and 0.05% chromium potassium sulfate in dH_20) or the electrostatic slides (Thermo Scientific™ Shandon™ ColorFrost™ Plus Slides; six sections per slide) in Everbrite Mounting Medium with 4',6-diamidino-2-phenylindole

(DAPI) for nuclear counterstaining (Biotium; Fremont, CA). The sole purpose of the DAPI staining was to visualize and distinguish subregions within the HPC.

In order to control for variability across experiments, one rat from each hormone condition was immunolabeled per batch. Additionally, to control for non-specific binding of the secondary antibody, each immunolabeling set had one negative control, where the sample was only treated with the secondary antibody.

A 3D image of the brain area of interest (30µm in depth) was captured using a Nikon Ti Eclipse inverted microscope equipped with 4x (NA 0.2) and 20x (NA 0.75) lenses, a Photometrics Evolve EMCCD camera and appropriate filter sets for DAPI and Alexa647.

Using Fiji (Schindelin et al., 2012), an image processing package by ImageJ, regions of interest within each image were selected and a mean intensity of the Alexa 647 immunoreactivity was obtained. The mean intensity of the Alexa 647 immunoreactivity was indicative of the relative amount of protein found in the corresponding sample.

Western Blot Analysis

A western blot analysis was performed to confirm the specificity of all primary antibodies used in the study. Given that the current study is identifying PRs in the female rat brain, all receptor antibodies were tested on female rat brain tissue. In addition, other organs were analyzed based on the rat RNA-Seq transcriptomic BodyMap (Yu et al., 2014; NCBI), showing the relative abundance of each progesterone receptor across organs and developmental stages in rats. Female rat brain tissue was compared to organs where specific PRs are most abundant to confirm specificity of each antibody. Thus, in addition to rat brain tissue, female rat lung tissue was analyzed for the mPRδ antibody. For the nPR antibody, rat

uterine and ovarian tissues were analyzed. For the mPRβ antibody, the brain had the highest contents of mPRβ, therefore no additional organs were analyzed.

A naturally cycling female Long Evans was euthanized via asphyxia by CO_2 followed by immediate decapitation. The brain was immediately removed, flash frozen using isopentane and dry ice and stored at -80°C. The brain was sectioned coronally and fresh dissections of the PFC, dSTR and HPC were acquired.

Tissues were sonicated in radioimmunoprecipitation assay (RIPA) lysis buffer (for nPR; Santa Cruz Biotechnology, CA, USA) or 2% SDS solution (for mPRβ and mPRδ) in 10mM Tris-HCl (pH 6.8) with protease inhibitor cocktail (Sigma). Proteins were centrifuged at 16, 438 x g for 30 minutes at 4°C and quantified by the bicinchoninic acid (BCA) protein assay method (BCA kit from ThermoFisher Scientific). 80ug of protein were separated by electrophoresis on an 8% SDS-PAGE gel for nPR, 10% gel for mPRδ and a gel for mPRβ at 100V. Proteins were transferred to a nitrocellulose membrane at 100V for 1 hour at 4°C. Membranes were blocked with 5% non-fat dry milk in 0.1M tris-buffered saline (TBS) with 0.1% polysorbate 20 (Tween 20 [T20]) for one hour at RT. Membranes were incubated in primary antibody overnight at 4°C. Primary antibody dilutions for each progesterone receptor-type were the same as described in the immunohistochemistry section. Blots were then washed in TBS-T20 three times for five minutes each and incubated in secondary goat anti-rabbit horseradish peroxidase (HRP) conjugated super-clonal antibody (1:5000; Thermofisher Scientific) for one hour at RT. Blots were washed in TBS-T20 three times for five minutes each and incubated in an enhanced chemiluminescence solution (ECL Substrate Kit [High Sensitivity]; Abcam, Toronto, ON) for one minute. Membranes were imaged using the Amersham 600 imager (GE Healthcare, QC, Canada).

Data Analysis

Immunohistochemistry is a semi-quantitative technique that can measure the relative antibody fluorescence and provide a visualization on how the protein is distributed across the tissue. Each microscope image used equivalent settings. A 3D image of a 30µm brain section of the PFC, dSTR and dHPC were analyzed using Fiji. First, the image was converted from a 3D image to a 2D by averaging all the DAPI and Alexa647 pixels on the z-plane. Next, an outline was drawn around areas of interest within each image (e.g., the CA1 region of the dHPC; based on Paxinos and Watson [1998]) and the mean receptor IR intensity of the highlighted area was obtained. Six sections per rat was analyzed to represent the entirety of each brain region. Therefore, the final mean obtained is the average immunoreactivity of the progesterone receptor in a single rat's brain region of interest.

Statistical Analysis

This experiment was a between-subjects design with a continuous dependant variable, the mean receptor IR intensity in arbitrary units. Thus, to determine whether there was a difference in receptor IR, across all groups, a one-way analysis of variance was conducted for each receptor type and brain region of interest. Finally, eta-squared (η^2) was reported in lieu of effect sizes.

Results

Experiment 1: Progesterone and memory systems bias

A chi-square test was performed to determine whether there was a memory bias within each hormone condition. Rats treated with E2+P (1 hr) were significantly more likely to use

response memory (χ^2 = 4.167, p = 0.04123) than place memory. The low E2 and high E2+P (4 hr) group showed a trend towards the use of response memory. The high E2 group and the high E2+P (15 min) showed a trend towards the use of place memory (Figure 3); however, differences in place and response memory in the last four groups did not reach statistical significance.

There was no significant difference between high and low E2 groups in memory bias; however, an odds ratio indicates that the use of place memory was 2.78 times higher in rats receiving high E2 than rats treated with low E2.

To determine whether P had an effect on memory bias, the high E2 group was compared to each of the high E2+P groups. There was a significant difference in memory bias between high E2 rats and high E2+P (1 hr) rats (χ^2 =5.371, p=0.02), showing that the proportion of rats using response or place memory was reversed in these two conditions. The OR indicates that the use of response memory was 4.05 times higher in rats receiving high E2+P (1 hr) than rats treated with high E2 alone. Though there was no statistically significant difference in memory bias between the high E2 and high E2+P (4 hr) group, the OR indicates that the use of response memory was 2.78 times higher in rats receiving high E2+P (4 hr) than rats treated with high E2. There was no significant difference in memory use between the high E2 and the high E2+P (15 min). The OR was 0.833 indicating that there was close to an equal chance of rats using place or response memory in either group. Therefore, high E2 along with long-term treatment of P reverses the effects of high E2 alone on memory bias.

Experiment 2: PR distribution in the HPC

Western blot images confirmed that each antibody used immunoreacted with a protein that migrated at the molecular weights of each receptor of interest: both nPR isoforms (expected at 90 kDa and 120 kDa) were detected between 150 kDa and 75 kDa, mPRβ (expected at 40 kDa) and mPRδ (expected at 38 kDa) were both detected at between 37 kDa and 50 kDa (Figure 4). IF images of the hypothalamus also confirmed nPR antibody sensitivity insofar as there was abundant IR in this brain region (Figure 5A) and that there was no significant effect of the hormone condition on nPR-IR in the hypothalamus (Figure 5B).

IF images revealed that mPRβ-IR were found in the pyramidal cell layer of the CA1, CA2, CA3, and DG of the HPC (Figures 6A). Similarly, IF images of nPR-IR were found in the same regions of the HPC (Figure 7A). There was little to no mPRδ-IR found in the principal/pyramidal cell layer of the HPC. Instead, mPRδ was localized in other layers of the HPC called the stratum lacunosum-moleculare (SLM) of the CA1 region and the hilus of the DG (Figure 8A). Thus, the SLM and hilus were analyzed instead. Analysis of variance showed there was no significant effect of hormone condition on intensity of IR for nPR, mPRβ nor mPRδ (Figures 6B-8B) and effect sizes were also small for all three receptor types.

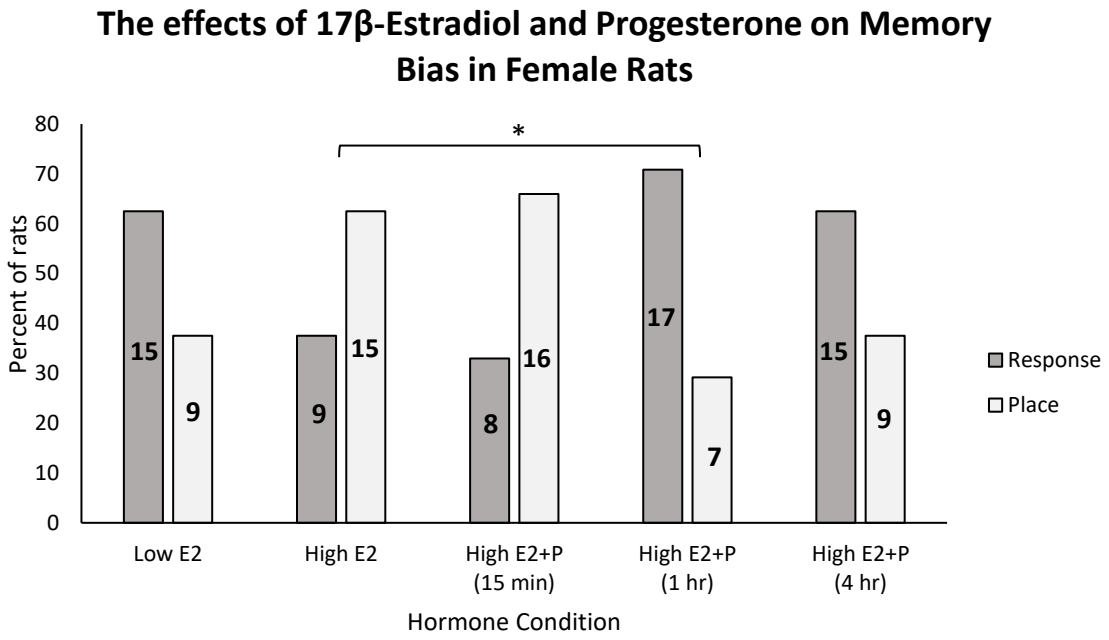


Figure 3. Proportion of ovariectomized rats using either place or response memory in low E2, high E2 and high E2+P conditions. High E2 significantly differed from high E2+P (1 hr) in memory bias (*p=.041). Numbers in bars represent the number of rats using that memory system.

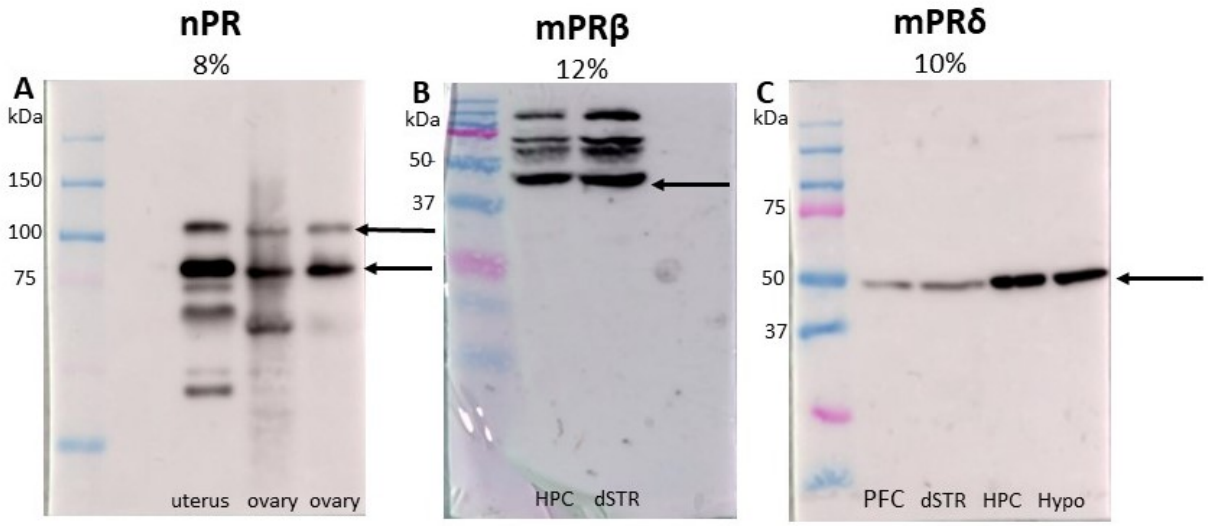
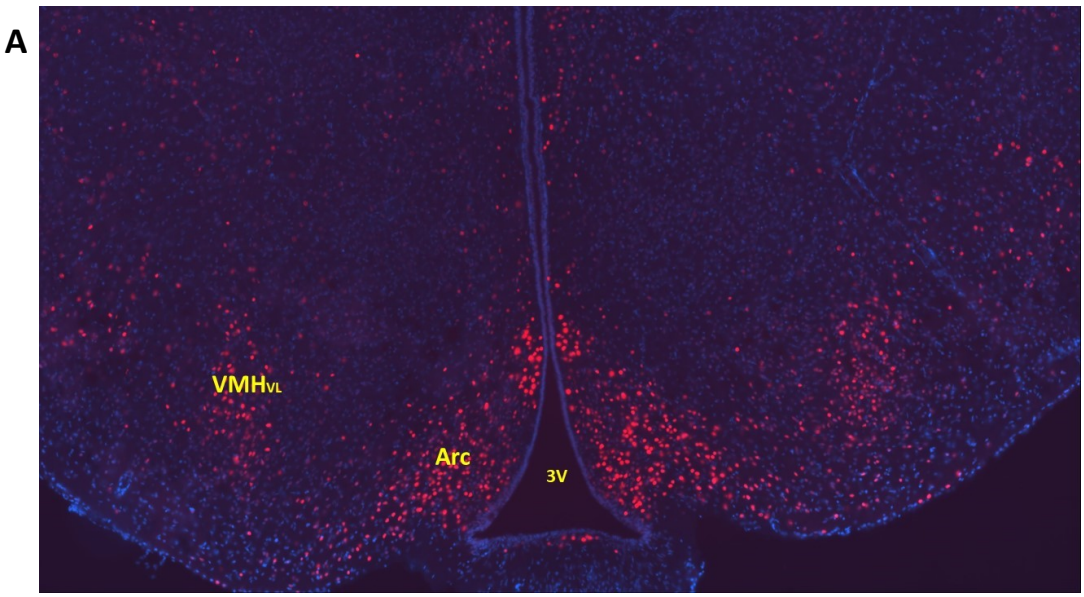


Figure 4. nPR, mPRβ and mPRδ detected by chemiluminescence (see arrow[s]) along with other non-specific proteins. PFC., prefrontal cortex. dSTR., dorsal striatum. HPC., hippocampus. Hypo., Hypothalamus.



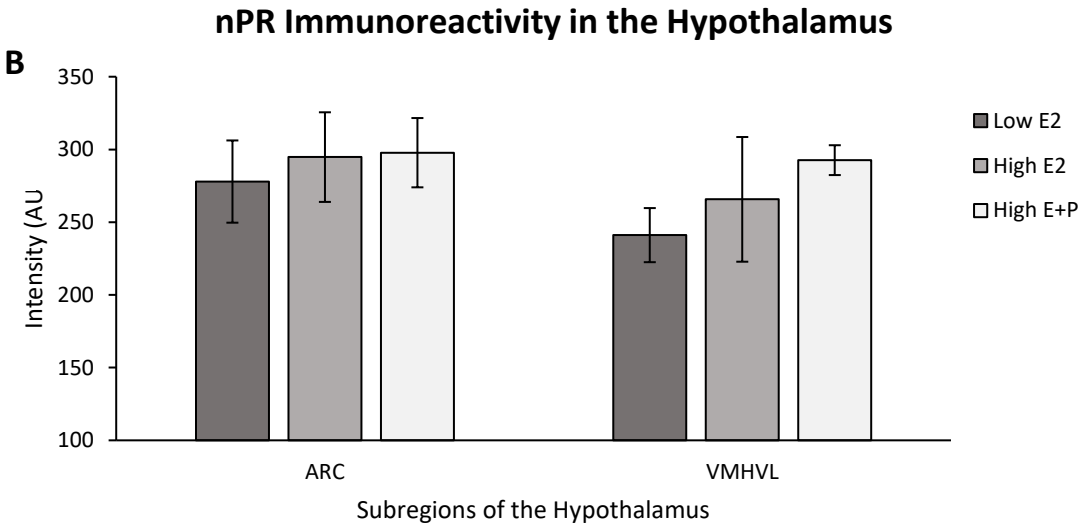
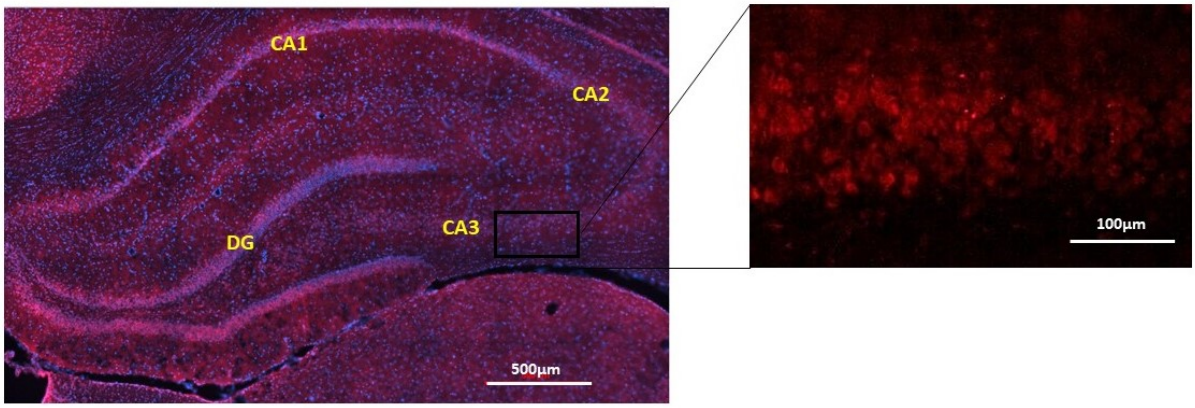


Figure 5. A, Immunoreactivity for nPRs (red) and DAPI stained neuronal cell nuclei (blue) in the VMHVL and ARC. B., There was no significant effect in mean intensity of nPR immunoreactivity across conditions in the ARC and VMHVL of the hypothalamus. 3V., Third ventricle. AU., Arbitrary units. ARC., Arcuate nucleus. VMHvl., the ventrolateral part of the ventromedial hypothalamus.

A



B

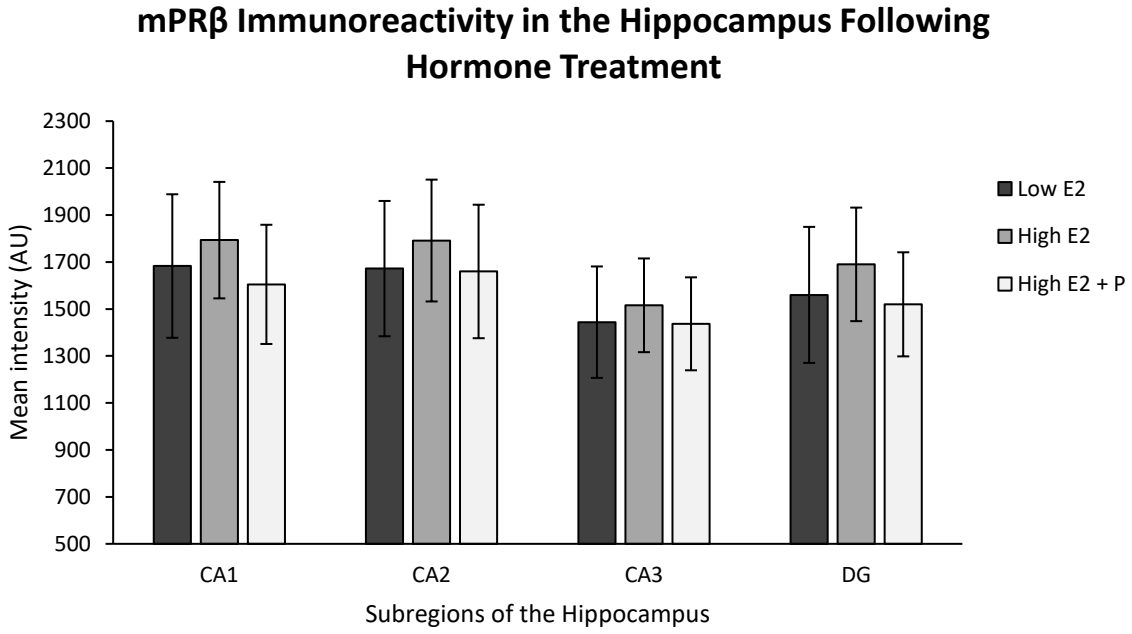
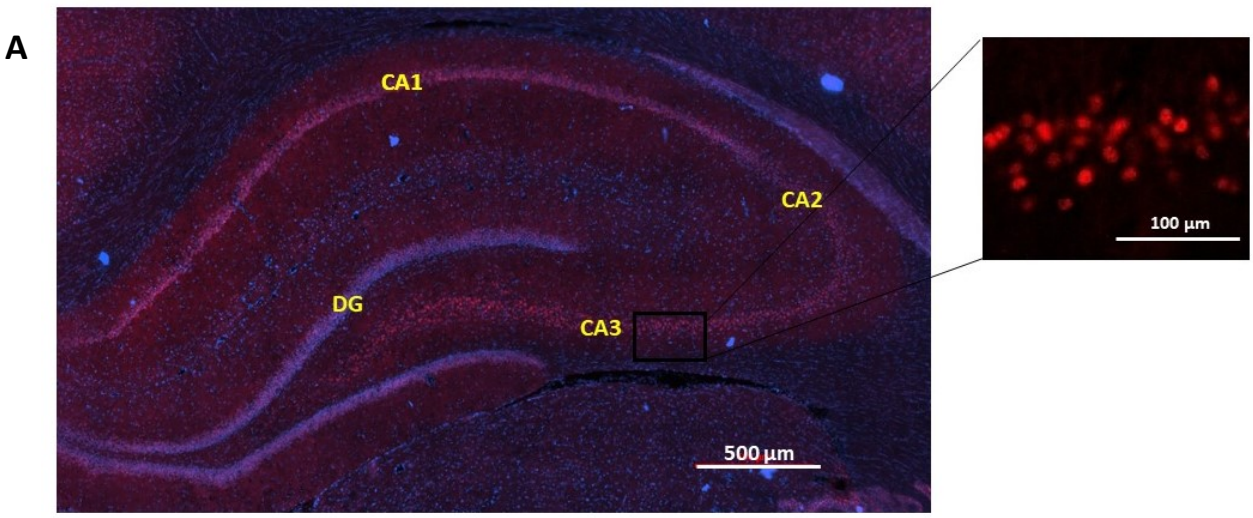


Figure 6. A, Immunoreactivity for mPRβ (red) and DAPI stained neuronal cell nuclei (blue) in the CA1, CA2, CA3 and DG of the HPC. Inset image without DAPI to improve visualization. B, mPRβ immunoreactivity of female rats that received low E2 (n= 6), high E2 (n= 6) or high E2 + P (n= 6). There was no significant effect in mean intensity of mPRβ immunoreactivity across conditions in the CA1, CA2, CA3 and DG of the HPC. AU., Arbitrary units. HPC., Hippocampus.



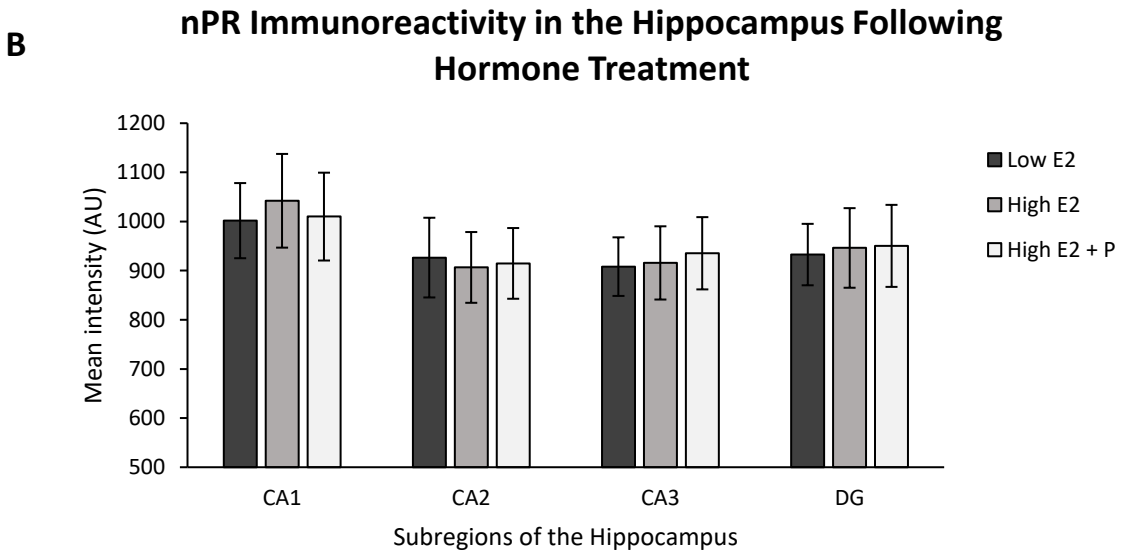
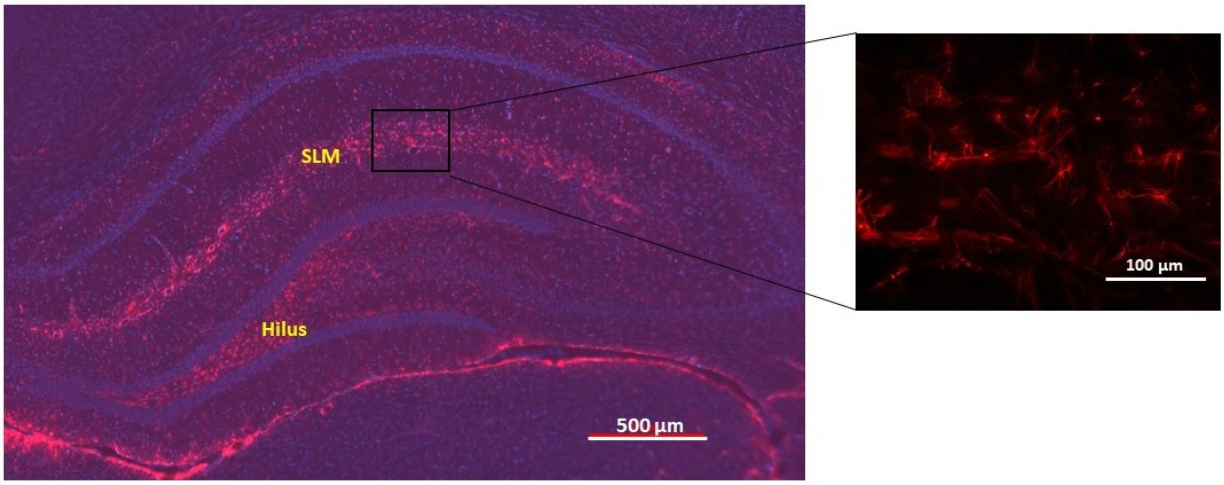


Figure 7. Immunoreactivity for nPRs (red) and DAPI stained neuronal cell nuclei (blue) in the CA1, CA2, CA3 and DG of the HPC. Inset image without DAPI to improve visualization. B, nPR immunoreactivity of female rats that received low E2 (n= 6), high E2 (n= 6) or high E2 + P (n= 6). There was no significant effect in mean intensity of nPR immunoreactivity across conditions in the CA1, CA2, CA3 and DG of the HPC. AU., Arbitrary units. HPC., Hippocampus.

A



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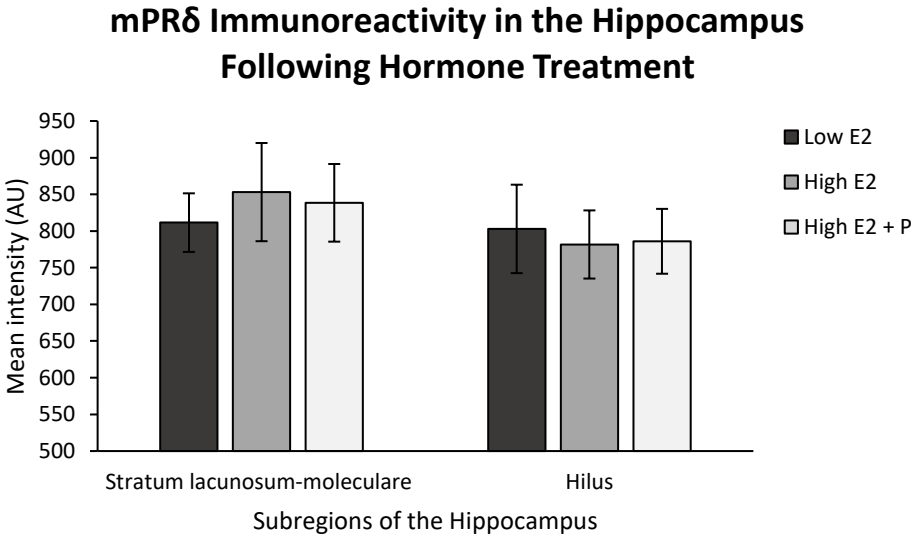

Figure 8. A, Immunoreactivity for nPRs (red) and DAPI stained neuronal cell nuclei (blue) in the SLM and hilus of the HPC. Inset image without DAPI to improve visualization. B, mPRδ immunoreactivity of female rats that received low E2 (n= 6), high E2 (n= 6) or high E2 + P (n= 6). There was no significant effect in percent volume of mPRδ immunoreactivity across conditions in the stratum SLM of the CA1 region and the hilus of the DG. AU., Arbitrary units. SLM., Stratum lacunosum-moleculare. HPC., Hippocampus.

Discussion

The objective of this study was two-fold. The first experiment aimed to determine whether P had an effect on memory bias in the modified plus maze. This experiment showed that P did affect memory bias by reversing the effects of high E2 when rats receive P one hour prior to testing. Secondly, to better understand the mechanisms by which P operates, antibodies directed at nPR, mPRβ and mPRδ were examined in the HPC. The effects of low E2, high E2, and high E2+P were examined on IR to these receptors in the HPC. The experiment established the presence of mPRβ, mPRδ, and nPRs in the female rat HPC and were found to be E2-insensitive in the HPC.

Experiment 1: Progesterone and memory systems bias

Results of experiment 1 confirm that high E2 bias female rats towards the use of hippocampal-mediated place memory, while low E2 biases rats towards the use of striatum-mediated response memory. Though no statically significant differences were detected between the low E2 and high E2 groups, the odds ratio shows a trend that is consistent with previous studies (Almey et al., 2014; Hussain et at., 2013; Korol & Kolo, 2002; Korol et al., 2004; Quinlan et al., 2008, 2013).

The high E2+P (15 min) and the high E2 alone groups both showed a bias towards place memory. Thus, the addition of P 15 minutes prior to the probe test did not have an effect on memory bias. The 15 minute subcutaneous injection prior to the probe test may not allow enough time for P to be absorbed into the bloodstream, cross the blood brain barrier, and affect neuronal transmission in time to induce behavioral changes. Though rapid effects, within 8 minutes were observed with ALLO (Johansson et al., 2002), perhaps this does not apply to P

administration. Conversely, the high E2+P (1 hr) group showed a significant bias towards response memory and was found to be significantly different from the high E2 alone group. A trend towards the use of response memory was also seen for the high E2+P (4 hr) group, however it was non-significant. On the other hand, the odds ratio showed that there was an effect. Therefore, according to the odds ratio, high E2 along with long-term treatment of P reverses the effects of high E2 alone on memory bias. As such, P provides striatum-mediated response memory a competitive edge over hippocampal-mediated place memory. Though the shift to response memory is not necessarily an impairment of place memory, it is consistent with previous studies showing that P impairs spatial memory performance in female rats and mice (Warren & Juraska, 1997; Chesler & Juraska, 2000; Harburger et al., 2007). The mechanism through which a reversal in memory bias occurs has yet to be determined. Genomic effects via nPRs can occur within hours to days, while non-genomic effects via mPRs occur within minutes to hours (Gellersen et al., 2008; Wilkenfeld et al., 2018). Since P had an effect when given one or four hours prior to testing, neither of these mechanisms can be ruled out without targeting specific PR types.

Experiment 2: PR distribution in the HPC

To explore the potential mechanism of how P influences memory bias, the distribution and regulation of three PRs were examined in the HPC. Results of experiment 2 confirms the IR of mPRβ, mPRδ and nPRs in the female rat HPC. Pang *et al*. (2013) had previously confirmed the presence of mRNA expression of these receptors in the human HPC. This is the first study to provide a visual of the IR of these receptors within the different subregions of the HPC.

Images of the hypothalamus were captured as a positive control for nPR antibody sensitivity. Images showed strong IR in the ARC and VMHVL of the hypothalamus (Figure 5A), suggesting that the antibody used here is capable of detecting nPRs. In addition, western blot analysis showed two single bands at the appropriate weights, suggesting that this antibody is selective to PR-A and PR-B isoforms of nPR.

Nuclear PRs were localized in the pyramidal cell layer of the CA1, CA2, CA3 and DG of the HPC. Based on previous studies (Parsons et al., 1982; Waters et al., 2008; Bali et al., 2012), it was hypothesized that nPRs in the CA1 region of the HPC would be E2-inducible, thus showing higher IR in the high E2 group and a lower IR in the low E2 and high E2+P groups. Results showed that hormone treatment did not have an effect on nPRs intensity in all the subregions of the HPC; nPRs had similar intensity throughout the pyramidal cell layers. All studies previously showed that nPR expression increases with E2 treatment when comparing OVX groups with no hormonal replacement to an OVX+E2 treatment group. In this study all rats were treated with E2. Therefore, nPRs may be E2-sensitive to both high and low levels of E2. To confirm this, results would need to be compared to an OVX group of rats with no hormone replacement. Guerra-Araiza *et al.* (2003) showed that the content of nPRs in the HPC was increased with E2 treatment and decreased following P administration. Again, an OVX group was compared to an OVX+E2 group and rats were treated with supraphysiological doses of P, about 8 times more than the current study. An OVX group was not included in this study because the hormone conditions were meant to mimic the hormonal profiles of intact female rats during the estrous cycle. Rats receiving low E2 would mimic the E2 levels of the estrus phase of the estrous cycle and the high E2 was administered to mimic the E2 levels in the early

proestrus phase and the high E2+P would mimic the late proestrus phase. With that in mind, the results of this study do support previous findings that showed that nPR expression in the HPC does not change during the estrous cycle (Guerra-Araiza et al., 2000; Guerra-Araiza et al., 2003). This could explain why the effects of E2-sensitive nPRs were previously only observed in OVX rats that received hormone treatment and not in naturally cycling rats.

The current study also showed that the hormone treatments did not have a differing effect on nPR IR in the hypothalamus (Figure 5B). An effect in the hypothalamus may have been observed if the two nPR isoforms were observed individually. Within the hypothalamus, gene expression of PR-B in the hypothalamus throughout the estrous cycle has been shown to be E2-sensitive, while PR-A is not (Guerra-Araiza et al., 2000). On the other hand, protein content was also similar across all phases of the estrous cycle for PR-B, while PR-A protein levels were significantly lower in the diestrus phase (Guerra-Araiza et al., 2003). Thus, when observing the expression of both isoforms together, as was done here, the hypothalamus did not show changes in nPR-IR across the hormone conditions. The antibody used here does not distinguish PR-A and PR-B, thus the findings here reflect the collective IR of both.

Like the nPRs, mPRβ-IR was localized in the pyramidal cell layer of the CA1, CA2, CA3, and DG of the HPC. These findings confirm the regional distributions of mPRβ reported by Intlekofer *et al.* (2011b) and Zuloaga *et al.* (2012). This study is the first to investigate whether mPRβ has E2-sensitive properties. Intlekofer and Petersen (2011a) showed that mPRβ mRNA is E2-sensitive in the anteroventral periventricular nucleus of the hypothalamus, and the sexually dimorphic nucleus of the preoptic area, but not in the VMHvl of the hypothalamus. Results from this study show that hormone treatment did not have an effect on mPRβ intensity in the

subregions of the HPC; mPRβ had similar IR throughout the pyramidal cell layers. Therefore, mPRβ may not be involved in the fast acting effects of P on hippocampal-mediated place memory. Frye *et al*. (2013) found no significant effects on open field, social interaction and elevated plus maze behaviour when infusing female rats with antisense oligodeoxynucleotides in the lateral ventricles targeted against mPRβ. Therefore, mPRβ, may not be involved in hippocampal-dependent cognitive behaviors. To date, there is only one study that provides insight on mPRβ's role in the CNS. Kasubuchi *et al.* (2017) found that mPRβ promotes P-dependant neurite outgrowth in PC12 cells in mice. PC12 cell lines are typically used in research relating to neurodegeneration-related pathologies. Thus, mPRβ may provide a neuroprotective role rather than a role in spatial/place memory.

The pyramidal cell layer, where nPR and mPRβ-IR were localized, has been implicated in spatial memory, episodic memories, and provides excitatory glutamatergic output to other cortical and subcortical regions (Klausberger & Somogyi, 2008). Kainic acid lesions in the CA3 region can impair spatial working memory in the radial arm maze (Handelmann & Olton, 1981), while colchicine lesions have also shown to impair spatial tasks (Xavier et al., 1999). It is hypothesized that the CA1 acts as a "novelty detector", detecting mismatches between cortical information concerning the current situation, with the stored predictions arriving from CA3 (Martin & Clark, 2007). This novelty signal might then result in the updating of stored information to eliminate the mismatch. Thus, the CA1, CA3 and DG subfields play a pivotal role in spatial memory and potentially other non-spatial behavioral paradigms.

Finally, mPRδ was expressed in the SLM layer of the CA1 region and the hilus of the DG. This study is the first to identify the presence of mPRδ in the female rat HPC. The SLM is part of

the temporoammonic pathway, which has direct afferent projections from layer III of the entorhinal cortex to the SLM layer of the CA1 region. The SLM mostly composed of GABAergic interneurons that regulate pyramidal cell activity in the CA1 hippocampal region (Khazipov et al., 1995; Capogna, 2011). Inhibitory GABAergic interneurons are also concentrated in the hilus. Hilar interneurons modulate the excitatory activity of granule neurons in the DG. This excitatory/inhibitory balance is thought to be needed for normal learning and memory (Andrews-Zwilling et al., 2012). Thus, the presence of mPRδ in the SLM and hilus suggests P4 may contribute to non-genomic, rapid effects or modulating excitatory activity in the HPC via inhibitory GABAergic interneurons. Results also showed that hormone treatment did not have an effect on mPRδ-IR in the subregions of the HPC; mPRδ had similar IR throughout the SLM and hilus. Thus, mPRδ may not be involved in the fast acting effects of P on hippocampal-mediated place memory, but perhaps other cognitive behaviors.

Allopregnanolone

Impairments in spatial performance have been linked to the GABAergic system. Female mice treated with P only or in conjunction with E2 had performed poorly in a T-maze footshock task compared to mice treated with E2 alone. When mice were administered with a GABA antagonist, picrotoxin, the progesterone-induced impairment was reversed, suggesting that P or its metabolites facilitate these effects through GABA binding (Farr et al., 1995). A derivative of P, ALLO, is a potent agonist for $GABA_A$ receptors and also fluctuates across the estrous cycle in the HPC, hypothalamus and midbrain (Frye et al., 2001). ALLO has also been found to inhibit spatial learning in the Morris water maze in male rats when treated with P eight minutes before testing (Johansson et al., 2002). Additionally, Murphy & Segal (2000) found that the conversion

of P to ALLO is necessary to decrease hippocampal spine density in rats. The enzymes responsible for producing ALLO have been identified in the hippocampus (Escudero et al., 2012). Therefore, the conversion from P to ALLO is another potential pathway by which P influences memory bias.

Ovarian hormones have also been implicated in neuroprotection in neurodegenerative diseases and TBIs. OVX rats that received E2 or P replacement showed a reduction in brain edema following a TBI compared to OVX rats with no hormone replacement (Shahrokh et al., 2009). P has been shown to reduce brain edema, inflammation and oxidant activity (Guennoun et al., 2015). It is hypothesized that these effects may be mainly mediated by ALLO. ALLO is a positive allosteric modulator of the $GABA_A$ receptor and one mechanism by which ALLO can exert neuroprotective effects by inhibiting neuronal apoptosis via the $GABA_A$ receptor. ALLO levels have also been shown to decline in patients with neurodegenerative diseases (Luchetti et al., 2011). These neuroprotective actions of P and its metabolite ALLO can be mediated by mPRs, specifically mPRδ. Unlike the other mPRs, mPRδ has a high binding affinity for P and ALLO has been shown to be an effective agonist (Pang et al, 2013). Thus, mPRδ may also have a neuroprotective role in the HPC.

Memory bias and PRs outside the HPC

The dSTR and mPFC are also involved in memory bias. In the modified plus maze, an infusion of E2 into the mPFC caused female rats to predominantly use place memory, while sham infusion rats were more likely to use response memory (Almey et al., 2014). In the brain, nPR mRNA expression has been identified in layer II and III of the mPFC in perinatal male and female rats (Willing & Wagner, 2016). P administration has been shown to increase $GABA_A$

receptor αI subunit mRNA expression in the mPFC (Andrade et al., 2012). Furthermore, an E2 infusion into the dSTR prior to training led female rats to perform more poorly on a response learning task (Zurkovsky et al., 2011). In the dSTR, Maclusky and McEwen (1980) identified the presence of nPRs using photo affinity labeling, while Parsons et al. (1982) reported that the caudate putamen was devoid of any E2-inducible and uninducible nPRs in female rats. The presence of all five mPRs and nPR mRNA expression has been identified in the human brain (Pang et al., 2013). Thus, the effects of P on memory bias may be occurring in other areas of the brain involved in memory bias, such as the dSTR and mPFC.

Methodological considerations

A western blot analysis was conducted to confirm the specificity of all primary antibodies used in the study. The molecular weights of all three antibodies were found to be within their expected range. However, the antibodies against mPRβ and nPRs showed additional protein content at other molecular weights (Figure 4). Thus, these antibodies were also binding to other proteins. Still, this does not mean that the antibodies were non-specifically binding during the immunohistochemistry procedure. Denatured proteins have more epitopes accessible during a western blot procedure than a folded protein during immunohistochemistry. Nevertheless, some of the antibodies may still have non-specific staining either with proteins of the same molecular weight or proteins of different molecular weights. More experiments (e.g., with peptide blocking, knockout tissue or transfected cell line) would be required to confirm this.

To determine whether nPR, mPRβ and mPRδ are expressed in the HPC, the study used immunofluorescent labeling, which allows the discrete localization of proteins to be observed. However, this technique is semi-quantitative; The IR does not directly equate to the actual quantity of PRs. The IR signal for each receptor is amplified as multiple primary antibodies will bind to the antigen and multiple secondary antibodies will bind the primary antibody. Additionally, the protein level of each receptor cannot be compared to one another due to the different blocking techniques and dilutions used to visualize the signals from each receptor.

Variances in mean intensity were observed across sets of immunolabeling in samples within the same hormone condition. These variances can be due to non-specific binding of antibodies to tissue (Figure 4A and 4B), residual plasma serum during perfusion, or a rat's hormone levels from individual pharmacokinetic differences. However, within each set, some showed an E2-sensitivity pattern (i.e., the high E2 rat had the high mean intensity, while low E2 and high E2+P had lower mean intensity values) while other immunolabeling sets did not show this pattern across the samples in each hormone condition. Therefore, despite the variance in mean intensity values across the sets of immunolabeling, our results support the conclusion that all three PRs were not sensitive to E2 in the HPC.

Conclusion

The effects of low E2 and high E2 on memory bias in this study remained consistent with previous rodent literature. The addition of P to high E2, one hour prior to testing, reversed the effects of high E2 on memory bias. Thus, both E2 and P bias female rats to use one memory system over the other in the modified plus maze. The findings support the growing evidence that place memory impairments from P administration in rodents may be due to the promotion

of GABAergic activity via ALLO. This experiment showed that nPR, mPRβ, and mPRδ are localized in the HPC. Both nPR and mPRβ were observed in the CA1, CA2, CA3 and DG of the HPC, while mPRδ was observed in the SLM of the CA1 region and the hilus of the DG. The presence of these receptors suggests that P can exert both genomic and non-genomic effects in the HPC. The IR of these PRs was not affected by ovarian hormone treatment, suggesting that these PRs in the HPC may have functions other than that of memory bias. However, other areas involved in memory bias (i.e., the dSTR and mPFC) remain to be examined further.

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Appendix A: Effect sizes for Experiment 2

PRs	*η2*
nPR	
CA1	0.0079
CA2	0.002381
CA3	0.005406
DG	0.001959
mPRβ	
CA1	0.016071
CA2	0.009077
CA3	0.005588
DG	0.016253
mPRδ	
SLM	0.019663
Hilus	0.006443