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Dissociable involvement of estrogen receptors in perirhinal cortex-mediated object-place memory in male rats

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ABSTRACT

Estrogens and the estrogen receptors (ER) - ERα, ERβ, and the G-protein coupled estrogen receptor (GPER) - are implicated in various forms of hippocampus (HPC)-dependent memory. However, the involvement of ER-related mechanisms in perirhinal cortex (PRh), which is necessary for object memory, remains much less clear. Moreover, there is a paucity of data assessing ER contributions to cognition in males,despite documented sex differences at the cellular level.We hypothesized that estrogens in PRh are important for object memory in males, assessingthe role of 17-βestradiol (E2), ERα, ERβ, GPER, and their downstream signaling pathways, in PRh-mediated object-in-place (OiP) memory in gonadally-intact male rats. Intra-PRh administration of E2 enhanced both long-term memory (LTM; 24 h) and short-term memory (STM; 20 min). Conversely, aromatase inhibition with letrozole impaired LTM and STM. The semi-selective ER inhibitor ICI 182780 impaired LTM, but not STM. This effect may be due to inhibition of ERβ, as the ERβagonist DPN, but not ERαagonist PPT, enhanced LTM. GPER was also found to be necessary in PRh, as the antagonist G15 impaired both LTM and STM. Western blot analyses demonstrated that phosphorylation levels of the extracellular signal-related kinase (ERK2 isoform), awell-establisheddownstream signaling pathway activated by estrogens through ERα/ERβ, was elevated in PRh 5 min following OiP learning.We also reportincreased levels of c-Jun N-terminal kinase (JNK; p46 and p54 isoforms) phosphorylation in PRh 5 min following learning,consistent with recent research linking GPER activation and JNK signaling in the HPC. This effect was abolished by intra-PRh administration of G15, but not letrozole, suggesting that JNK signaling is triggered via GPER activation during OiP learning, and is possibly E2-independent, similar to findings in the HPC. These results, therefore, reveal interesting dissociations between the roles of various ERs, possibly involving both estrogen-dependent and independent mechanisms, in PRh-mediated object-place learning in male rats.

1. Introduction

The involvement of estrogens and the estrogen receptors (ER) ERα, ERβ, and the G-protein coupled estrogen receptor (GPER), in **female** rodent long-term memory (LTM) is well-established (Frick et al., 2015). Furthermore, several studies have demonstrated rapid (i.e., non-genomic) mnemonic effects of ER activation, indicating possible roles in short-term memory (STM) (Ervin et al., 2013). Much of this literature has focused on hippocampus (HPC)-dependent forms of object recognition tasks. Indeed, both systemic and intra-HPC administration of 17-β estradiol (E2) have been shown to enhance long-term and short-term object recognition memory in ovariectomized (OVX) female rodents (rats and mice) (Fernandez et al., 2008; Frye et al., 2007; Luine et al., 2003; Pereira et al., 2014; Phan et al., 2015, 2012). Conversely, blocking local E2 synthesis by inhibiting aromatase (the enzyme that catalyzes the conversion of testosterone to E2) impairs long-term object memory in female mice (Tuscher et al., 2016). Similar to the effects of E2, systemic and intra-HPC ERα, ERβ, and GPER agonists can significantly improve memory in these tasks in female mice and rats (Jacome et al., 2010; Kim et al., 2016; Lymer et al., 2017; Pereira et al., 2014; Phan et al., 2015, 2011), whereas intra-HPC GPER antagonism impairs long-term object memory (Kim et al., 2016) and ERα/β

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antagonism blocks the long-term object memory enhancing effects of E2 (Fernandez et al., 2008). At the mechanistic level, several studies have demonstrated that in OVX female rodents, E2 infusion into the HPC significantly increases phosphorylation (P) of the cell signaling kinase extracellular signal-related kinase (ERK) (Fan et al., 2010; Fernandez et al., 2008; Kuroki et al., 2000; Pereira et al., 2014). Furthermore, ERK activation has been shown to be necessary for E2-induced memory consolidation (Fan et al., 2010; Fernandez et al., 2008), and ERα/β antagonism blocks E2-induced P-ERK, linking the pro-mnemonic effects of E2 to activation of ERα and/or ERβ and subsequent ERK signaling (Fernandez et al., 2008). Conversely, the role of GPER in HPC-dependent forms of long-term object memory **in mice** has recently been linked to c-Jun N-terminal kinase (JNK), rather than ERK, phosphorylation, and this effect is seemingly independent of E2-induced cellular and mnemonic effects (Kim et al., 2016).

Although typically thought of as female hormones, estrogens (and ERs) play a significant role in male brain development (Maclusky and Naftolin, 1981). Sex differences have been noted in the synaptic and cell-signaling events that occur following ERα/β activation or E2 influence (exogenous or locally synthesized) in the HPC **(**Jain et al., 2019; Koss et al., 2018; Oberlander and Woolley, 2016; Vierk et al., 2014A few studies have investigated estrogens and ERs in HPC-dependent object recognition tasks in male rats and mice. Specifically, systemic E2 has been shown to improve short-term and long-term object memory in males (Jacome et al., 2016; Koss et al., 2018), while preliminary data suggest that aromatase inhibition causes mnemonic impairments (Koss et al., 2017). Unlike female mice, the effects of E2 on P-ERK within the HPC were not seen in male mice, nor was P-ERK found to be necessary for E2-induced memory enhancements (Koss et al., 2018).

Many studies indicate that, in rats (mice may be different; Cohen et al., 2013**),** the HPC is chiefly implicated in processing spatial information about objects, whereas perirhinal cortex (PRh) is necessary to process object identity, irrespective of spatial location (Barker and Warburton, 2011; Forwood et al., 2005; Piterkin et al., 2008; Winters et al., 2004). In fact, these regions are doubly dissociable in the rat brain for spatial and object information, respectively (Winters et al., 2004), and engage distinct epigenetic and molecular mechanisms for successful object memory consolidation (Fonseca et al., 2013; Mitchnick et al., 2016a, 2015). The involvement of estrogens and ERs in PRh-mediated object memory is not well established. In one series of studies using OVX female rats, intra-PRh/entorhinal cortex infusions of either E2 or the ERβ agonist DPN, enhanced novel object preference (Gervais et al., 2016, 2013). Moreover, E2 replacement in female OVX mice facilitated object memory, in addition to increasing c-fos immunoreactivity, an index of cellular activity, in PRh (Fonseca et al., 2013). C-fos positive cell counts were significantly higher in the object learning group compared to the context-only group (Fonseca et al., 2013), suggesting that E2 in PRh enhances cell activity, gene expression, and/or protein synthesis, and subsequently object recognition memory. These studies indicate that, similar to the HPC, estrogens and estrogen receptors function to facilitate PRh-mediated object memory. However, these few experiments have demonstrated mnemonic enhancements using agonists, only indicating an *involvement* of estrogens and ERs in PRh-mediated object memory, not a necessity. Additionally, the involvement of downstream signaling cascades, such as ERK and JNK, has not been investigated in PRh. As PRh-mediated object memory appears to rely on many of the plasticity mechanisms demonstrated to be influenced by estrogen signalling, such as long-term potentiation and long-term depression (Banks et al., 2014), further investigation into the possible mechanistic basis for estrogen-mediated modulation of PRh-based memory is warranted.

In the current study, we assessed the involvement and necessity of estrogen, ERs, and downstream signaling cascades in PRh-mediated short-term and long-term object-in-place (OiP) memory, using gonadally-intact male rats. Under a similar premise to the spontaneous object recognition (SOR) task, during the OiP task rodents explore four different novel objects in a unique configuration during a sample phase. Following a variable retention delay, the rodent is re-exposed to these four objects, but two of the objects have switched locations. Rodents preferentially explore the displaced objects if they recognize the original object-place configurations, and this effect is dependent on PRh, HPC, and prefrontal cortex (PFC) (Barker and Warburton, 2011; Mitchnick et al., 2016a, 2015). The associative nature of the OiP task makes it a more accurate model of human episodic memory than the SOR task, and its reliance on an established network of brain regions means that any effects observed in the present study in PRh can be evaluated in future within the HPC and PFC.

Intra-PRh administration of various pharmacological agents allowed us specifically to target PRh during memory consolidation. Based on previous literature assessing similar questions in PRh in females (Gervais et al., 2016, 2013), we predicted memory enhancement following intra-PRh administration of 17-β estradiol or an ERβ agonist in males. Contrary to the effects of E2, we expected inhibition of local estrogen synthesis to impair memory. Although local estrogen synthesis in PRh has not been assessed previously, it has been shown to occur in the human temporal lobe, possibly including PRh (Steckelbroeck et al., 1999), as well as the male rat HPC (Hojo et al., 2004). Furthermore, estrogen levels remain high in the HPC of male rats following castration (Hojo et al., 2009). ERα has not been detected in PRh of rats; therefore, we predicted no effect of ERα agonism. We also predicted that GPER antagonism would impair PRh-dependent memory, given that the GPER has been localized to PRh (Hazell et al., 2009) and similar effects have been found in the HPC (Kim et al., 2016). Accordingly, we expected that learning would increase ERK phosphorylation in PRh, given the consistent involvement of this signaling cascade in HPC-dependent memory (Fernandez et al., 2008; Kelly et al., 2003; Pereira et al., 2014). Conversely, we were unsure whether JNK signaling would be involved in PRh-dependent memory; however, given the link between GPER activity and JNK activation we predicted that if GPER was found to be necessary in PRh-mediated memory, JNK would be phosphorylated following learning and that this would be abolished if GPER was inhibited.

2. Methods

2.1. Subjects

Collectively, 43 adult male Long Evans rats were used for behavioural testing (Charles River, New York). All rats weighed between 300-325 g at the start of testing (approximately 3 months of age), were pair-housed in opaque cages, and maintained on a 12 -h reverse light cycle (8:00pm lights on). All behavioural testing occurred during the rats' waking hours (dark phase), although the testing room was illuminated by ceiling-mounted fluorescent lighting, as is typical for such vision-mediated tasks (Ennaceur and Delacour, 1988; Winters et al., 2008**)**. During experimental testing periods, each rat received 20 g of rodent chow to maintain an 85-90% free-feeding body weight to enhance exploratory behaviour in the task (Mitchnick et al., 2015; Winters and Bussey, 2005; Winters and Reid, 2010**)**. On experimental testing days rats were fed after testing was completed. Food restriction is a standard method used to increase exploratory behaviour **(**Bussey et al., 1999; Mitchnick et al., 2015; Winters and Bussey, 2005; Winters and Reid, 2010**)**. Water was available *ad libitum*. All procedures adhered to the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee at the University of Guelph.

2.2. Surgical Procedures

All rats underwent intracranial surgical cannula implantation targeting PRh (Mitchnick et al., 2016a, 2015). Prior to surgery, rats were anesthetized using isoflurane (Benson Medical Industries, Markham,

Table 1
Drug and intra-PRh infusion parameters.

Agent	Action	Dose	Vehicle	Infusion Timing	Min. Days Between Infusions	Parameters Derived From
E2 (17-β Estradiol)	Estrogen (ligand)	2.5 μg/μl	10% DMSO	Immediate Pre-sample	2d	(Fernandez et al., 2008; Walf and Frye, 2007); pilot data, see S1.1, S2.1, Fig. S1
Letrozole	aromatase inhibitor	9.0 μg/μl	70% DMSO	20 min pre-sample	13d	(Mitchnick et al., 2016b; Mitwally and Casper, 2001; Sato and Woolley, 2016)
ICI 182780	ERα/β antagonist	0.1 μg/μl	10% DMSO	Immediate Post-sample	1 week	(Long and Nephew, 2006; Mitchnick et al., 2016b)
PPT	ERα agonist	0.5 μg/μl; 2.5 μg/μl	50% DMSO	Immediate Post-sample	2d	(Chang et al., 2009; Gervais et al., 2016; Mitchnick et al., 2016b; Walf and Frye, 2007)
DPN	ERβ agonist	0.5 μg/μl; 2.5 μg/μl	10% DMSO	Immediate Post-sample	2d	(Chang et al., 2009; Gervais et al., 2016; Mitchnick et al., 2016b; Walf and Frye, 2007)
G15	GPER antagonist	0.04 μg/μl	50% DMSO	Immediate Post-Sample	4d	(Duarte-Guterman et al., 2015; Kim et al., 2016); pilot data

Ontario), and received a systemic subcutaneous injection of the analgesic meloxicam (5 mg/ml; Boehringer Ingelheim). With the incisor bar placed at -3.3 mm, rodents were secured in a stereotaxic frame (Koft Instruments, Tujunga, CA). The scalp was incised and retracted to expose the skull. Holes were drilled into the skull and 22-gauge indwelling cannula guides were implanted according to the following coordinates relative to the skull at Bregma (Paxinos and Watson, 1998): anteroposterior, -5.5 mm; lateral, ± 6.6 mm; dorsoventral, -7.0 mm. The guide cannulas were anchored to the skull by four jeweller screws and dental acrylic. Dummy cannulas, 0.36 mm in diameter, were placed into the guide cannulas where they remained at all times except during infusions. Dummy cannulae and infusors extended 1.1 mm past the end of the guide cannula. Post-surgery the scalp was sutured. All animals were given 7-10d to recover before the start of behavioural testing.

2.3. Drugs and Infusions

The drugs, action, vehicle, and infusion parameters are listed in Table 1. Each compound was dissolved in the relevant vehicle (e.g., 10% DMSO) in 0.9% physiological saline. A minimum of 2d were left in between trials to ensure no carry-over effects from the infused agents. Longer inter-trial delays were used for specific compounds depending on their mode of action, half-life, or our pilot data (Table 1). All infusions were delivered by a Harvard Apparatus (Hilliston, MA) precision syringe pump set to administer infusions at a rate of 0.5 μl/min for 2 min. Two, 1 μl Hamilton syringes delivered the infusate (1 μl) through propylene tubing and infusors inserted in the intra-PRh indwelling cannulas. The infusors were left in place for an additional 1.5 min to allow for the diffusion of the infusate. Dummy cannulas were returned following each infusion. Infusions were performed in the rats' colony room under red light.

2.4. Object-in-place (OiP) Procedure

The behavioural procedures and apparatus used have been described previously (Mitchnick et al., 2016a, 2015). Briefly, all rats experienced two habituation sessions on successive days, in which they explored an empty open field (23in x 23in x 23 in. for 5 min, followed by a mock microinfusion (no infusate). Actual microinfusions occurred at different time points relative to the sample phase, depending on the compound (Table 1). To determine whether a compound impaired memory, rats explored four different novel objects in an open field arena during a 5-min sample phase. A 24 h delay was used to assess LTM, which a 20 min delay was used to assess STM. In one case (G15 administration), we used a longer STM delay of 2 h due to the time course of action of the drug (see Sections S1.3, S2.2, and Fig.S2 for explanation). During the 2-min choice phase, rats viewed the same four objects, but two objects had switched locations (right or left, counterbalanced) creating a 'novel side' (Fig. 1). Preference for the objects on the novel side was interpreted as being indicative of memory for the original object-place configuration. To determine whether a compound enhanced memory, the sample phase was reduced to 3 min to assess LTM, and 2 min to assess STM (Fig. 1). In these 'sub-optimal' learning versions of the tasks, a rat receiving a control infusion will not demonstrate memory. All behavioural experiments were conducted using a within-subjects design in order to reduce variance and the number of animals used. More specifically, each experiment consisted of two trials where rats received a vehicle infusion in one trial and an experimental infusion in the other trial, counterbalanced. Rats saw a new set of four novel objects during each trial. A minimum of 1d was left between trials to avoid potential object confusion.

2.5. OiP Data Analysis

The sample and choice phases were recorded by a ceiling mounted camera, and exploration, scored by an experimenter blind to the treatment conditions, was defined as directing the nose to the objects at a distance of < 2 cm and/or touching it with the nose. Novelty preference in the choice phase was used as an indicator of memory and was determined by the amount of novel-object exploration (N) and familiar-object exploration (F) in the following discrimination ratio (DR): DR = (N-F)/(N + F). Although the total 2 min of choice exploration was recorded and scored, DRs were calculated from the exploration levels in the first minute only, during which object discrimination is typically most robust (Dix and Aggleton, 1999). When multiple related experiments were run in the same rats, a repeated-measures analysis of variance (ANOVA) was employed, with drug (inhibitor/activator, control) and retention delay (20 min/2 h, 24 h) as our independent measures, and DR as our dependent measure. Further paired-samples t-tests were run as planned comparisons between drug and control conditions within each testing phase. *A priori* one-sample t-tests were also applied to each drug condition to determine whether memory differed significantly from chance. These two complementary statistics allow us to determine if the drug manipulation abolished memory (i.e., comparing to a DR of 0), in addition to assessing whether this is significantly different from control performance. Partial eta squared effect sizes were also computed for all ANOVAs, using SPSS. Cohen's d effect sizes were calculated for paired t-tests. On several occasions, different cohorts of rats were used for complementary experiments. In these cases, an ANOVA was not computed; rather, the above mentioned within-subjects t-test and one-sample t-tests were employed. A list of the experiments performed on each cohort of rats can be found in Table S1. For all experiments, total sample and choice object exploration levels were examined in each testing phase to assess potential pre-existing or drug-induced non-mnemonic differences in general exploratory behaviour between conditions, using paired t-tests. Control analyses were non-significant unless otherwise reported. Means and standard errors are reported in Table S2. All analyses were conducted as two-tailed tests

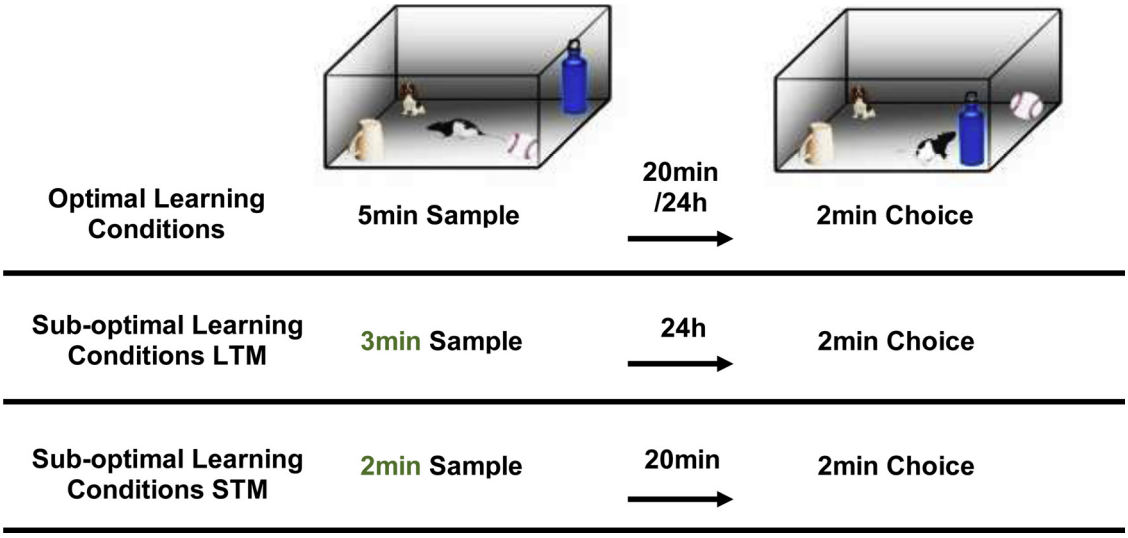

Fig. 1. Object-in-Place task parameters. Schematic of the OiP task and running parameters with a representative object set. Two objects are switched during the choice phase, which vehicle rats will preferentially explore. With a 5-min sample phase, rats successfully remember 24 h later (Optimal Learning Conditions). This allows the assessment of memory-impairing agents. When the sample phase is reduced to 3 min and 2 min, rats do not remember 24 h and 20 min later, respectively, allowing for the assessment of memory enhancing agents.

with a confidence interval of 0.05. Multiple comparisons were Bonferroni-corrected.

2.6. Protein Isolation and Western Blot Procedure

Forty-eight adult male Sprague Dawley rats (Charles River, New York) received intra-PRh surgical cannula implantation (as described in Section 2.2). Following recovery and habituation to the apparatus (as in Section 2.2, 2.4), rats were assigned to one of four groups ($n=$ 12/group). The non-learning (NL) group received a vehicle microinfusion (70% DMSO) 20 min ($n=$ 6) or 2 h ($n=$ 6) prior to empty-apparatus exploration. The learning (L) group received a vehicle microinfusion (70% DMSO) 20 min ($n=$ 6) or 2 h ($n=$ 6) prior to a sample phase (section 2.4). The learning + G15 (L + G15) group received a microinfusion of G15 (0.4 μg/μl) 2 h prior to a sample phase. The learning + letrozole (L + let) group received a microinfusion of letrozole (9.0 μg/μl) 20 min prior to a sample phase. The drug doses and infusion parameters paralleled the behavioural experiments (Section 2.3; Table 1). Although 50% DMSO was used as a vehicle in the G15 experiments, 70% DMSO used in the letrozole experiments was chosen for all groups to ensure that effects were not due to varying amounts of DMSO. The timing of infusions in relation to the sample phase, either 20 min or 2 h prior, was kept consistent with behavioural experiments (i.e., G15 and letrozole experiments), and half of the vehicle infusions adopted either parameter. G15 was infused 2 h prior to learning because it is unknown exactly when G15 begins to take effect, but our results (Section 3.7) demonstrate that it exerts effects at 2 h post-infusion. In all groups, 5 min following empty-apparatus exploration or sample phase, rats were briefly exposed to CO_2 and their brains quickly removed. The entire, bilateral PRh was excised, placed in a tube, and immediately frozen on dry ice (Mitchnick et al., 2016a, 2015). Tissue was maintained at −80 °C until further processing.

Tissue samples were homogenized in protein lysis buffer (1% Triton X-100, 50 mM Tris, 150 mM sodium chloride, pH 7.5) with added DNase I (700 units/mL), protease inhibitors (1 mM AEBSF, 10 μM leupeptin, 25 μM aprotinin, 10 μM pepstatin A) and Na_3VO_4 (5 μM). The homogenates were placed on ice, briefly sonicated, given 20 min to rock on ice for protein isolation, and centrifuged two times at 17,530xg for 15 min at 4 °C. Protein concentrations were determined using a Bradford assay (Bradford, 1976), and lysates were stored in small aliquots at −20 °C until western blotting was performed. The protocol for western blotting has been described previously (Mendell et al., 2016). Briefly, 25 μg of total protein for each sample was loaded onto 10 % sodium dodecyl sulfate polyacrylamide gels, and electrophoresis was performed using Mini-PROTEAN Tetra cells (Bio-Rad, Ontario, Canada). The Trans-Blot SD Turbo transfer apparatus (Bio-Rad) was used to transfer proteins onto 0.45 μM nitrocellulose (Bio-RaD), with transfer conditions of 30 min at 25 V (constant voltage). Following the transfer, membranes were briefly rinsed in tris-buffered saline with 0.1% Tween-20 (TBS-T) and blocked in either 5% non-fat milk, 5% bovine serum albumin (BSA; Fisher Scientific) or a combination of milk and BSA for 1.5 h. Membranes were rinsed twice for 5 min in TBS-T and incubated on an orbital shaker overnight at 4 °C in primary antibody for phospho-ERK1/2 (1:1000; Cell Signaling Technologies/CST), ERK1/2 (1:1000; CST), phospho-JNK (1:1000, CST), JNK (Santa Cruz Biotechnology), or alpha-tubulin (1:500,000; Sigma-Aldrich). Blots were rinsed twice for 5 min in TBS-T, incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit IgG (1:2500; Cell Signaling) in milk or BSA, then rinsed twice for 5 min and twice for 10 min in TBS-T. Proteins of interest were visualized with Luminata Forte HRP substrate (Millipore Canada) on a ChemiDoc MP imaging system (Bio-Rad). Densitometry was performed using Image Lab v4.1 software (Bio-Rad).

2.7. Western Blot Data Analysis

Two-way ANOVAs and Tukey-Kramer post-hoc tests were performed for all western blot densitometric quantitative analysis, with the exception of comparisons between the non-learning vs. learning groups within each ERK isoform, for which a two-tailed Student t-test (Bonferroni-corrected) was used in an *a priori* manner. Data were tested for normality using Komolgorov-Smirnov normality tests and for homogeneity of variance using Bartlett's tests. All statistical analysis for western blot data was performed with GraphPad Prism version 7.0 for Mac (GraphPad Software Inc). Statistical significance was set to $p <$ 0.05.

3. Results

3.1. Histology

All rats included in final behavioural analyses had guide cannulae

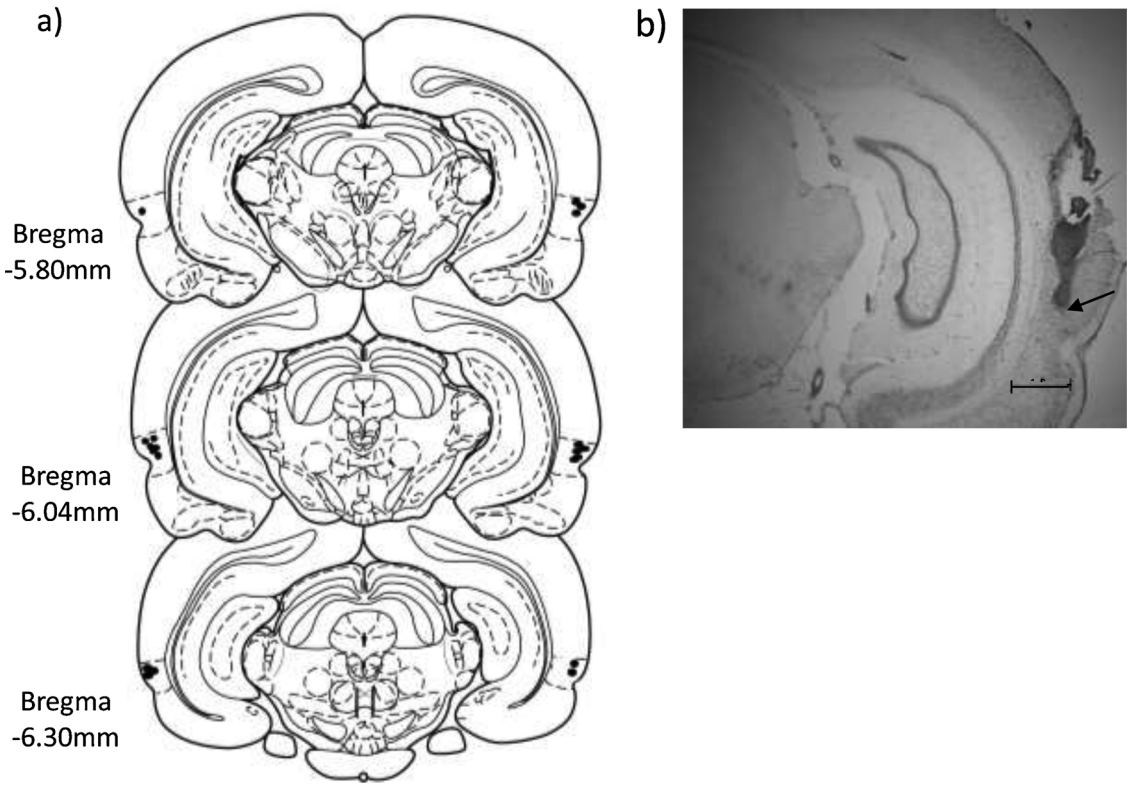

Fig. 2. Histology. Cannula placement verification. a) Schematic representation of infusion needle tip placement from one cohort of rats (cohort 4; see Table S1). These placements are representative of placements from all rats used in behavioural experiments. Cannulas were consistently located between 5.80 and 6.30 mm posterior to Bregma (Paxinos & Watson, 1998). b) Photomicrograph (1.25x magnification) of a Nissl stained section illustrating the tract of a PRh cannula. The arrow is indicating the tip of the infusor.

located bilaterally with infusion needle tips terminating in PRh (Burwell, 2001); these placements were consistently located at approximately 5.80 to 6.30 mm posterior to Bregma (Fig. 2a,b).

3.2. E2 Enhances STM and LTM

The memory enhancing effects of exogenously administered estrogen were assessed using E2 (Fig. 3a). When administered the vehicle solution, rats did not demonstrate a novelty preference greater than chance (t_{11} = -0.12, p = 0.918) at a 20 min delay, but they performed significantly above chance when given E2 (t_{11} = 5.86, p < 0.001) in the sub-optimal OiP task. Moreover, performance in the treatment conditions differed significantly (t_{11} = 4.89, p < 0.001, d = 1.98). In a separate set of rats, similar effects were found when a 24-h delay was employed; specifically, when administered the vehicle solution, rats **did** not demonstrate a novelty preference significantly greater than chance (t_{10} = 0.64, p = 0.539), but they did following E2 (t_{10} = 3.79, p = 0.004), and the treatment conditions differed significantly (t_{10} = 2.43, p = 0.038, d = 1.12). These results indicate that intra-PRh E2 enhances short-term and long-term OiP memory in male rats.

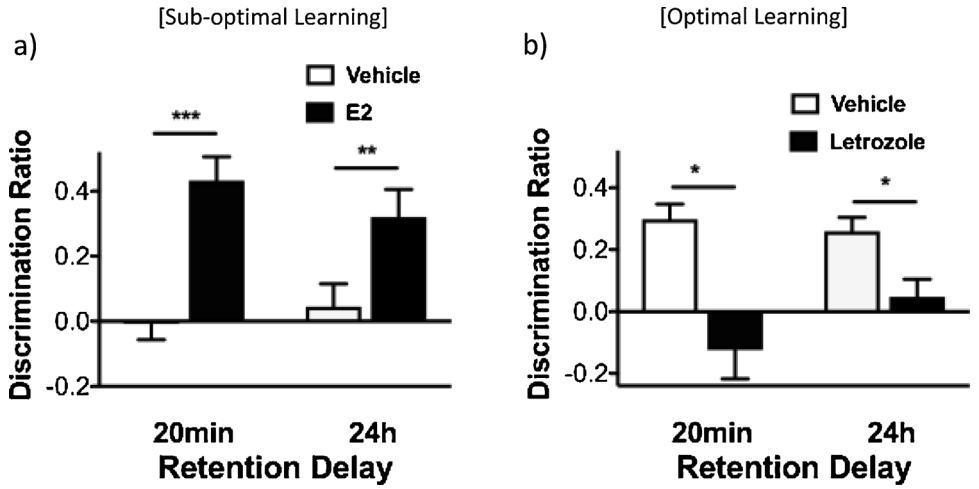

Fig. 3. Involvement of Estrogen in PRh-mediated OiP memory. a) Immediate post-sample administration of E2 enhanced STM (n = 12) and LTM (n = 11). b) 20 min pre-sample administration of the aromatase inhibitor letrozole, impaired STM **(n = 11)** and LTM (n = 10). Data are mean DR ± SEM. * p < 0.05

3.3. Letrozole impairs STM and LTM

The necessity of endogenous estrogen was assessed using the aromatase inhibitor, letrozole (Fig. 3b). When assessed 20 min following object learning, the letrozole condition did not perform significantly greater than chance (t_{10} = -1.33, p = 0.217), whereas the vehicle condition did (t_{10} = 6.11, p < 0.001). Moreover, performance in the treatment conditions differed significantly (t_{10} = 4.05, p = 0.003, d = 1.79). There was a significant difference between total choice exploration (t_{10} = 2.55, p = 0.031), but rats explored more in the drug condition (M = 14.23 s, SEM = 0.83 s) than the vehicle condition (M = 10.29 s, SEM = 1.23 s), and therefore it is unlikely that this caused the mnemonic impairment. In a separate set of rats, similar effects were found when a 24-h delay was used; specifically, when administered letrozole, rats did not remember significantly greater than chance (t_9 = -1.33, p = 0.217), but they did in the vehicle condition (t_9 = 5.76, p < 0.001), and performance in the treatment conditions also differed significantly (t_9 = 2.85, p = 0.020, d = 1.31). Thus, these results demonstrate that intra-PRh aromatase inhibition impairs short-term and long-term OiP memory in male rats.

3.4. ERα/β are necessary for LTM, but not STM

The necessity of ERα and ERβ were assessed using the ERα/β antagonist, ICI 182780 (Fig. 4a). A 2 × 2 repeated-measures ANOVA with treatment condition (ICI 182780, vehicle) and retention delay (20 min, 24 h) as independent variables, and DR as a dependent variable, did not produce a significant interaction term ($F_{1,8}$ = 4.11, p = 0.077, η_p^2 = 0.340). There was, however, a significant main effect of retention delay ($F_{1,8}$ = 57.19, p < 0.001, η_p^2 = 0.877), but no main effect of drug condition ($F_{1,8}$ = 3.44, p = 0.101, η_p^2 = 0.301). *A priori* t-tests demonstrated that rats in both the vehicle (t_8 = 4.12, p = 0.003) and drug (t_8 = 4.25, p = 0.003) conditions displayed a significant novelty preference with a 20-min retention delay, and no differences were apparent between conditions (t_8 = -0.06, p = 0.951, d = 0.04). When a 24 h delay was employed, only rats in the vehicle condition displayed significant novelty preference (t_8 = 8.10, p < 0.001 for the vehicle; t_8 = -3.14, p = 0.014 for drug condition, significantly below chance). Furthermore, a significant difference between treatment conditions was noted (t_8 = 8.04, p < 0.001, d = 3.47). These results suggest that ERα/β inhibition within PRh impairs long-term, but not short-term, OiP memory.

3.5. ERβ, but not ERα, agonism enhances LTM

To probe which of the classic ERs might be responsible for the LTM impairments caused by ICI 182780, we used selective ER agonists and assessed their ability to enhance LTM given a sub-optimal sample phase (Fig. 4b). At a low dose (0.5 μg/μl), the ERα agonist PPT did not enhance novelty preference; neither the drug condition (t_9 = -1.12, p = 0.288) nor the vehicle condition (t_9 = -0.69, p = 0.505) had novelty preference above chance, and performance between the treatment conditions did not differ (t_9 = 0.52, p = 0.618, d = 0.25). In a separate cohort of rats, a higher dose of PPT (2.5 μg/μl) generated similar results; neither the drug condition (t_{10} = -1.85, p = 0.093) nor the vehicle condition (t_{10} = 0.62, p = 0.552) displayed novelty preference. The drug and vehicle conditions differed significantly from one another (t_{10} = 2.50, p = 0.030, d = 0.77), but this is likely due to the very small variance displayed by both groups, rather than any meaningful group difference, given that neither group demonstrated memory from chance. Thus, ERα likely does not contribute significantly to PRh-mediated long-term OiP memory in male rats.

At a low dose (0.5 μg/μl), the selective ERβ agonist DPN did not enhance memory (Fig. 4c); neither the drug condition (t_9 = -0.41, p = 0.689) nor the vehicle condition (t_9 = 0.97, p = 0.360) displayed a novelty preference, and performance in the two conditions did not

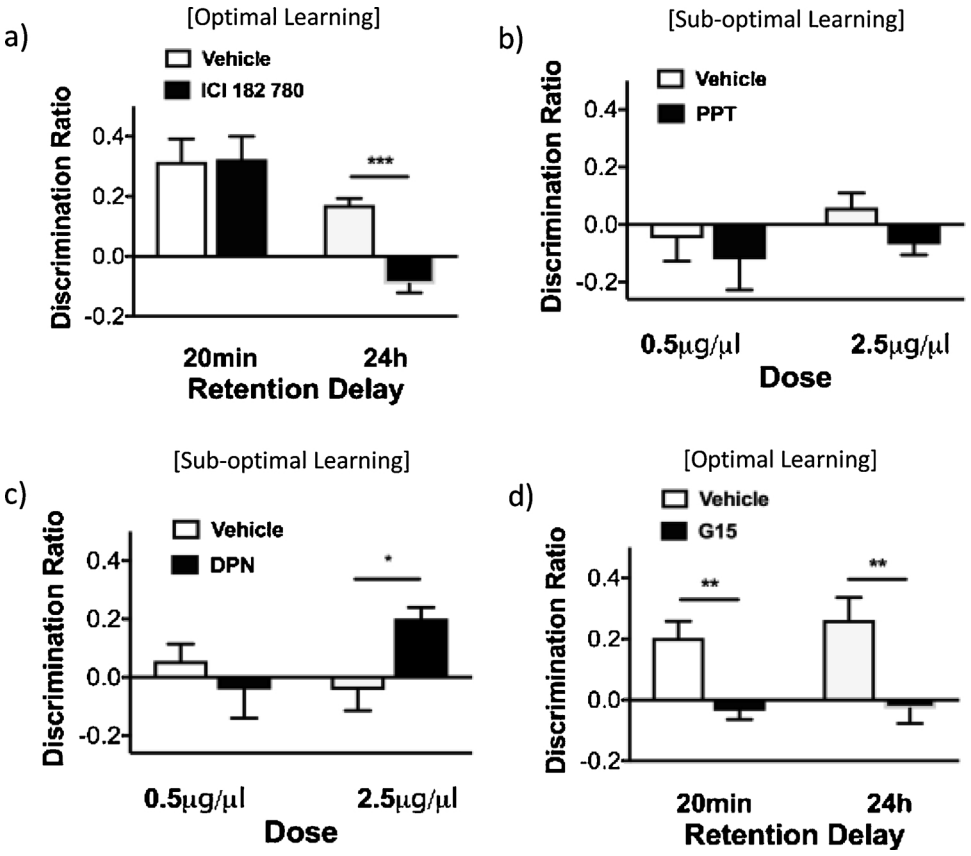

Fig. 4. Involvement of Estrogen Receptors in PRh-mediated OiP memory. a) Immediate post-sample administration of the ERα/β antagonist ICI-182-780 (n = 10), impaired LTM but not STM. b) To probe which ER(s) might be involved in the LTM-impairing effects of ICI-182-780, ER agonists were employed. Immediate post-sample administration of the ERα agonist PPT, did not enhance LTM at either the low dose (n = 10) or high dose (n = 11). d) Immediate post-sample administration of the higher dose (2.5μg/μl) of the ERβ agonist DPN, enhanced LTM (n = 11), where the lower dose did not (n = 10). d) Immediate post-sample administration of the GPER antagonist G15 (n = 12) impaired STM and LTM. Data are mean DR ± SEM. * p < 0.05; **p < 0.01; ***p < 0.001

differ from one another (t_9 = 1.23, p = 0.250; n = 10, d = 0.38). Conversely, in a different cohort of rats, a higher dose of DPN (2.5 μg/μl) enhanced memory; specifically, the drug condition had a novelty preference significantly greater than chance (t_{10} = 5.25, p < 0.001), but the vehicle condition did not (t_{10} = -0.61, p = 0.553); performance in the drug conditions also differed significantly from one another (t_{10} = 4.12, p = 0.003; n = 11, d = 1.02). Thus, ERβ activation in PRh can improve long-term OiP memory in male rats, and inhibition of ERβ may mediate the LTM impairment caused by ICI 182780. Interestingly, in our unpublished data we have noted the opposite pattern of effects in HPC-dependent OiP memory; specifically, PPT was found to enhance memory, while DPN had no effect (Mitchnick et al., 2016b). Not only are these results interesting in light of the functional differences between PRh and HPC (Brown and Aggleton, 2001), they also highlight the value of the OiP task as such differential results cannot be attributed to task differences, but rather the presumed differential functional contributions of the distinct brain regions

3.6. GPER is necessary for STM and LTM

The necessity of GPER was assessed using the GPER-selective antagonist G-15 (Fig. 4d). A 2 × 2 repeated-measures ANOVA with drug condition (G-15, vehicle) and retention delay (2 h, 24 h) as independent variables and DR as a dependent variable, revealed a non-significant interaction ($F_{1,11}$ = 0.13, p = 0.731, η_p^2 = 0.011). There was, however, a significant main effect of drug condition ($F_{1,11}$ = 88.27, p < 0.001, η_p^2 = 0.889), but no main effect of retention delay ($F_{1,11}$ = 0.25, p = 0.629, η_p^2 = 0.022). Accordingly, *a priori* t-tests indicated that only rats in the vehicle condition displayed a significant novelty preference when a 2 h delay was employed (t_{11} = 3.71, p = 0.005 and t_{11} = -1.26, p = 0.233 for the vehicle and drug condition, respectively). Moreover, these conditions differed significantly (t_{11} = 3.64, p = 0.004, d = 1.58). Similar results were found at the 24 h delay; specifically, only rats in the vehicle condition displayed a novelty preference significantly above chance (t_{11} = 3.52, p = 0.003 and t_{11} = -0.59, p = 0.570 for the vehicle and drug condition, respectively), and the conditions differed significantly (t_{11} = 3.28, p = 0.007, d = 1.33). These results indicate that the GPER-antagonist G-15, within PRh, impairs short-term and long-term OiP memory in male rats.

3.7. P-JNK is increased following learning, and this effect is abolished by G15 but not letrozole

A significant effect of treatment on JNK phosphorylation was detected using a two-way ANOVA ($F_{3,32}$ = 11.57, p < 0.0001; n = 5/group; Fig. 5b,c), with no significant differences in JNK isoform ($F_{1,32}$ = 1.73, p = 0.198) or interaction between treatment and JNK isoform ($F_{3,32}$ = 1.68, p = 0.191). Learning significantly increased JNK phosphorylation compared to the non-learning group (Tukey-Kramer, q_{32} = 7.05, p = 0.0001), and this effect was inhibited by G15 (learning vs. learning + G15; Tukey-Kramer, q_{32} = 5.161, p = 0.005), but not letrozole (letrozole vs. learning; Tukey-Kramer, q_{32} = 0.793, p = 0.943, and letrozole vs. non-learning; Tukey-Kramer, q_{32} = 4.369, p = 0.021). The learning-induced increase in JNK phosphorylation was still apparent 20 min following learning, compared to the non-learning group (Fig. S3b, S3c).

For ERK phosphorylation, no significant treatment ($F_{3,82}$ = 2.11, p = 0.106; n = 11-12/group), ERK isoform ($F_{1,82}$ = 2.09, p = 0.153), or interaction ($F_{3,82}$ = 0.445, p = 0.722) effects were detected by a two-way ANOVA (Fig. 5c, d). Given the previous findings that E2 increases ERK phosphorylation and that ERK activity was necessary for E2-induced object memory enhancements (Fernandez et al., 2008; Fortress et al., 2013; Kuroki et al., 2000), in addition to the finding that learning only can modulate P-ERK (Kelly et al., 2003), we expected to see a learning-induced enhancement in P-ERK; therefore, planned Student t-tests were run between the learning and non-learning groups for each ERK isoform. A significant increase in ERK2 phosphorylation was found following learning (t_{21} = 2.488, p = 0.021), whereas no effect was found for ERK1 phosphorylation (t_{21} = 1.186, p = 0.249). There was a small but significant decrease in P-ERK 20min following learning, compared to the non-learning group (Fig. S3d, S3e).

4. Discussion

The present findings indicate that intra-PRh E2 enhances, and inhibition of local E2 synthesis impairs, short-term and long-term OiP memory in male rats. Furthermore, at least one of the classic ERs (likely ERβ), as well as GPER, are necessary for long-term OiP memory, and GPER activation is necessary for short-term OiP memory. These results demonstrate the necessity of E2 and ERs in PRh-mediated object-place memory of male rats and implicate ER-related signalling pathways similar to those previously demonstrated in the HPC.

4.1. Mnemonic involvement of estrogens in PRh

E2-induced memory enhancement in male rats has been seen in a variety of tasks, including the Morris water maze, radial arm maze, alternating T-maze, and extinction of condition taste aversion (Heikkinen et al., 2002; Packard et al., 1996; Yuan and Chambers, 1999). Recently, intra-HPC E2 has also been found to enhance long-term object-location and SOR memory in male mice (Koss et al., 2018), similar to robust findings in the HPC of female rodents (Fernandez et al., 2008; Frye et al., 2007; Luine et al., 2003; Pereira et al., 2014; Phan et al., 2015, 2012). Here, we have demonstrated that a single, post-learning infusion of E2 within PRh, enhances both short-term (20 min) and long-term (24 h) OiP memory in gonadally-intact male rats. The involvement of E2 in PRh-mediated memory in females has been previously shown. Specifically, E2 replacement in OVX female mice was shown to increase the number of c-fos + cells in PRh, but not HPC, following a typical SOR sample phase, when compared to context-only controls (Fonseca et al., 2013). Furthermore, E2 was shown to enhance both short-term and long-term SOR memory when infused into PRh of OVX female rats (Gervais et al., 2016, 2013). The present findings demonstrate for the first time that E2 can similarly enhance PRh-dependent object memory consolidation in males.

Here, we also report mnemonic impairment following aromatase inhibition, as a single pre-sample intra-PRh infusion of letrozole impaired both short-term and long-term OiP memory. This demonstrates the necessity of brain-synthesized E2 for PRh-dependent object memory consolidation in the male rat, and is in accordance with previously documented memory impairments by systemic of intra-HPC aromatase inhibitors in HPC-dependent memory in male and female rodents (Dutertre and Smith, 2003; Graham and Milad, 2014; Koss et al., 2017; Mitchnick et al., 2016b; Tuscher et al., 2016; Zhao et al., 2018), as well as adult human females (Bayer et al., 2015; Shilling et al., 2003). Moreover, demonstrating a loss of function allows us to state more confidently that brain-derived estrogens are *necessary* for PRh-mediated memory consolidation, in comparison to the memory enhancing effects of E2. Interestingly, intra-HPC aromatase inhibition in gonadally-intact male rats has been shown to enhance water maze performance (Moradpour et al., 2006), but has no effect on object recognition in gonadally-intact male mice (Koss et al., 2017). Furthermore, systemic aromatase inhibition enhanced egocentric working memory in male, gonadally-intact rats (Alejandre-Gomez et al., 2007). However, Graham and Milad (2014) noted impairments in long-term fear extinction memory in gonadally-intact male rats following systemic aromatase inhibition, and we have also noted short-term object memory deficits in gonadally-intact male rats following intra-HPC aromatase inhibition (Mitchnick et al., 2016b). It is not clear what underlies these discrepant results, although they could be related to differences in species (rat vs. mouse) or task parameters.

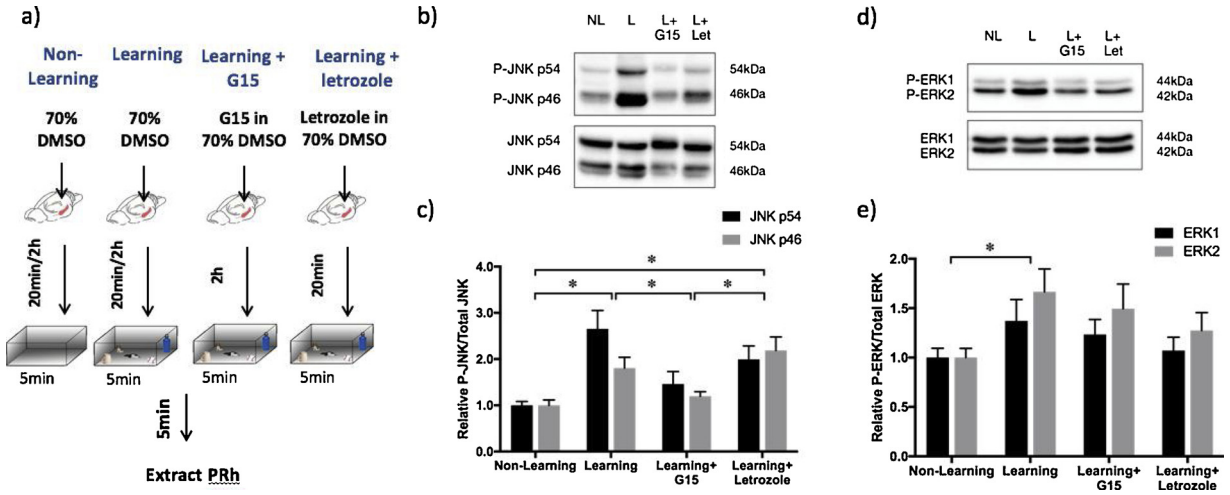

Fig. 5. Learning-induced Phosphorylation of ERK and JNK. a) Schematic of behavioural parameters. 70% DMSO was used as a vehicle for all infusions to ensure that any changes in P-ERK or P-JNK was not due to varying levels of DMSO. In the previous behavioural experiments, letrozole was infused 20 min pre-sample. Although G15 was administered immediately post-sample, a 2 h retention delay was employed due to the delayed onset of action. Therefore, to maintain consistency with our behavioural experiments, letrozole and G15 were infused at different time points pre-sample, in the western blot analyses. For the non-learning and learning groups, half of the rats received vehicle 2 h pre-sample, while the other half received vehicle 20 min pre-sample. b) Representative blots for P-JNK p54 and p46 (top; activated) and total JNK p54 and p46 (bottom). c) P-JNK (collapsed across isoforms) was enhanced following learning, compared to the non-learning group. This effect was abolished by G15, but not letrozole. d) Representative blot for P-ERK1/2 (top; activated) and total ERK1/2 (bottom). e) P-ERK2 was enhanced following learning, compared to the non-learning group (planned comparison). No other effects were present. [NL = non-learning; L = learning; Let = letrozole; data represent mean +/- SEM phosphorylated/total protein relative to the non-learning group. *$p < 0.05$.

4.2. Mnemonic Involvement of ERα, ERβ, and GPER in PRh

Using the semi-selective inhibitor ICI 182780, we have shown that the ERα and ERβ antagonism impaired long-term, but not short-term, OiP memory, when infused into PRh of gonadally-intact male rats. Given that ICI 182780's mechanism of action targets the genomic effects of ERα and ERβ (Dudley et al., 2000), it is perhaps not surprising that STM was unimpaired. Furthermore, the antagonistic actions of ICI 182780 on ERα and ERβ might be stronger or more prominent than the suggested GPER agonist effects (Tocris), given that GPER antagonism in PRh impaired memory (see below), suggesting that mnemonic enhancements would be seen with a GPER agonist. In order to determine whether the impairing effect of ICI 182780 might be mediated by one or both of the classic ERs, we assessed the mnemonic consequences of ERα and ERβ agonism, using PPT and DPN, respectively. Specifically, intra-PRh administration of DPN, but not PPT, enhanced long-term OiP memory. Given that ERβ, but not ERα, immuno-reactive cells were found in PRh of female mice (Merchenthaler et al., 2004), their effects are perhaps not surprising. Similarly, Gervais et al. (2016) found that ERβ agonism within PRh enhanced SOR memory in OVX female rats. Collectively, these results suggest that ERβ is involved in PRh-mediated LTM, in both sexes.

Evidence exists to suggest that ERα and ERβ can regulate both excitatory and inhibitory tone in the HPC (Frick et al., 2015; Phan et al., 2015). Interestingly, approximately 95% of ERβ+ neurons in PRh, regardless of gonadal status, colocalize with parvalbumin (PV)-containing GABAergic interneurons (Blurton-Jones and Tuszynski, 2002). This is not the case for the HPC, with the exception of the subiculum (major output source) (Blurton-Jones and Tuszynski, 2002). This suggests that ERβ activity in PRh induces inhibition. Interestingly, PRh-mediated object recognition memory relies on long-term depression (LTD), a form of synaptic 'weakening', with the supposition that the neural response decrement associated with successful recognition is reliant on LTD (Griffiths et al., 2008). It is possible that ERβ-mediated activation of PV-containing interneurons would inhibit post-synaptic excitatory neurons, leading to LTD and facilitating successful PRh-mediated long-term object memory. Indeed, Gervais et al. (2015) noted decreases in spine density of only mature spines in PRh (area 35) following E2 replacement in female OVX rats. The selective reduction of mature spines without changes in densities of other spine types indicates mature spine shrinkage, and is a process that occurs following low-frequency stimulation that induces LTD (Matsuzaki, 2007). In the current experiments, ICI 182780 and letrozole might have decreased learning-induced LTD and **increased** mature spine densities, leading to a memory impairment, while DPN and E2 could have had opposite effects.

In addition to determining the involvement of the classic ERs in PRh-mediated memory, here, we also show that the GPER is necessary. Intra-PRh administration of the selective GPER antagonist G15, impaired long-term and relatively short-term OiP memory, in male gonadally-intact rats. Many other groups have shown involvement of GPER in HPC-dependent memory in female rodents (Ervin et al., 2015; Gabor et al., 2015; Hammond et al., 2009; Hawley et al., 2014; Kim et al., 2016; Lymer et al., 2017), but to our knowledge, this is the first demonstration of a cognitive function for GPER in PRh, as well as in male rodents. This suggests that GPER may promote memory in multiple brain regions and in both sexes. Whether G15 would cause STM impairment with even shorter retention delays (i.e., less than 2 h) requires additional experimentation.

4.3. ER-related Intracellular Signaling in PRh Following Object Learning

Given the well-documented connection between estrogenic effects and ERK signaling in female mice (Boulware et al., 2013; Fan et al., 2010; Fernandez et al., 2008; Pereira et al., 2014), we assessed levels of ERK phosphorylation in PRh following learning. Specifically, 5 min following an OiP sample phase (i.e. object learning), P-ERK2 levels were enhanced in PRh when compared to context-only controls. We additionally noted a small but significant decrease in P-ERK 20 min following learning, which might represent a rebound effect. Kelly and colleagues (2003) also noted P-ERK (ERK1) increases in male, gonadally-intact rats 5 min after a typical SOR sample phase, although they did not note these increases in PRh (or CA1), but rather in the dentate gyrus subregion of the HPC (Kelly et al., 2003). It is possible that the use of four different objects in our OiP task, versus two identical objects in the SOR task that Kelly et al. (2003) used, produced a larger and

more detectable increase in P-ERK. Interestingly, recent research has shown that E2 does not increase HPC P-ERK in gonadectomized or intact male mice, as it does in female mice (Koss et al., 2018). This is in contrast to our findings and those of Kelly et al. (2003), who found elevations in P-ERK in male gonadally-intact rats. These results could be species-specific (rat vs. mouse), or due to procedural differences (e.g. brain region; following learning vs. estrogen administration). It is clear that further work needs to be done to determine the specifics with regards to estrogen-related ERK signaling in males.

Recent evidence has suggested that GPER signals through JNK, rather than ERK (Kim et al., 2016). Given that we found GPER to be necessary within PRh for OiP memory, we assessed JNK activation following learning. Specifically, 5 min following an OiP sample phase (object learning), P-JNK was increased in PRh of gonadally-intact male rats. This effect was still present 20 min following learning, whereas Kim et al. (2016) found P-JNK levels were only elevated 5 min following GPER agonism (G1), but not at 15 min or 30 min. Moreover, at the 5 min time-point, this increase was blocked by G15, but not letrozole, indicating that GPER activity is necessary for learning to increase P-JNK, but possibly in an E2-independent manner. Therefore, in the male rat PRh, learning appears to activate GPER, subsequently triggering the phosphorylation of JNK signaling, which seems to be necessary for PRh-mediated short-term and long-term object memory, as intra-PRh G15 impaired OiP task performance. Although we have not demonstrated the necessity of JNK signaling in PRh, other researchers have shown a requirement for GPER activated P-JNK in the HPC (Kim et al., 2016), and it is possible that the same mechanism operates in PRh; however, given the established functional differences between PRh and HPC, further research will be required to evaluate this possibility. These researchers additionally found that the E2-induced object memory and P-ERK enhancements were blocked by ERK inhibition, but not JNK inhibition, and suggested this as evidence that GPER, which signals through JNK, was not functioning as an ER in the HPC to regulate memory (Kim et al., 2016). There is some debate as to whether GPER is indeed an estrogen receptor (Levin, 2009), and in the present study, the finding that letrozole did not abolish the learning-induced increases in PRh P-JNK might also be taken as evidence that GPER can regulate memory in an estrogen-independent manner. However, there are numerous accounts of GPER-dependent effects of estrogens (Srivastava and Evans, 2013). Future studies may look to determine whether GPER antagonism can block the memory-enhancing effects of E2 in PRh.

Our demonstration that object learning significantly increases P-JNK in PRh of male rats, and that this effect is abolished with co-administration of G15, provides evidence for GPER-activated JNK signaling in PRh-mediated object memory, and fits with previous literature showing similar results in the HPC of female mice (Kim et al., 2016).

4.4. Conclusions

In the current study, we have determined that intra-PRh E2 enhances, and inhibition of local E2 synthesis impairs, short-term and long-term OiP memory in male rats. Moreover, the current data suggest that at least one of the classic ERs (likely ERβ), as well as GPER, are necessary for LTM, and that GPER is necessary for STM. Taken in tandem, our results demonstrate the necessity of E2 and ERs, specifically ERβ and GPER, in PRh-mediated object memory of male rats, and implicate similar ER-related signaling pathways in PRh as has been shown in the HPC. Additional work will be required to determine whether this estrogen influence generalizes to PRh-mediated performance in the spontaneous object recognition task for object identity. Previous work indicates that PRh manipulations that affect OiP-type tasks typically influence SOR task performance in the same way (Barker et al., 2007; Bussey et al., 2000) and it has been suggested that PRh contributions to these tasks are functionally similar (Brown et al., 2010). Thus, we predict that estrogens likely contribute to object recognition memory in a similar manner to that demonstrated here for the OiP task. On a related note, it should be emphasized that many of the findings implicating estrogens and ERs in object memory, whether in males or females, have been produced using some variation of the spontaneous exploration/novelty preference paradigm (including the OiP task used here). Other reports, using alternative procedures for assessing object memory (such as the delayed nonmatching to sample, DNMS, task for rodents) indicate that the neural bases for performance in these tasks might not always overlap, and this includes the potential role of estrogens. Indeed, previous studies have indicated that facilitative estrogenic effects observed in the novelty preference-based SOR task might not extend to DNMS object memory performance, at least in female rats; in fact, these researchers reported impairing effects of both systemic and intra-PRh E2 in the DNMS task despite enhancements in novel object preference (Gervais et al., 2016, 2013). Other seemingly contradictory results have been noted, such as the impairing and enhancing effects of aromatase on different forms of memory in male rodents, as discussed above (Alejandre-Gomez et al., 2007; Koss et al., 2017; Moradpour et al., 2006; Wee et al., 2014). Future work will be essential to evaluate the generalizability of the present results to other types of object and non-object memory task performance in males and females, and the influence of peripheral versus brain-synthesized estrogen.

The finding that learning alone, as opposed to following exogenous E2 or ER agonism, can lead to enhancements in P-ERK and P-JNK provides additional, compelling evidence that these signaling pathways are implicated in typical memory consolidation, and not just to enhance memory beyond normal boundaries. Future work might look to determine whether similar mechanisms operate in the HPC and PFC for OiP memory, a task which requires PRh, HPC, and PFC contributions (Barker and Warburton, 2011), enabling direct regional comparisons without the potential confounds of task differences. Moreover, this task has been shown to be sensitive to sex differences following various manipulations (Cost et al., 2012; Howland et al., 2012), indicating that a replication of the current experiments is warranted in female rats. Regardless, the present results indicate unequivocally that ER-related mechanisms operate within PRh of male rats to influence object-place information processing.

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The authors declare no competing financial interests.

Contributions

KAM, ALM, EC, NJM, BDW designed the experiments; KAM, ALM, CEW, KJ, SDC, AMM ran the experiments; KAM, ALM, EC, NJM and BDW wrote the manuscript.

CONFLICT OF INTERST

None of the authors were employed by any company or institution outside of the University of Guelph during the conceptualization, data collection, writing, or submission of this article. The authors declare no conflict of interest, monetary or otherwise.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.psyneuen.2019.05.005.

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