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Investigation of the Role of the G-Protein-Coupled Estrogen Receptor in Memory Consolidation in Gonadectomized Male Mice

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by

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ABSTRACT

INVESTIGATION OF THE ROLE OF THE G-COUPLED PROTEIN ESTROGEN RECEPTOR IN MEMORY CONSOLIDATION IN GONADECTOMIZED MALE MICE

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Estrogens are cholesterol-derived hormones that play crucial physiological and pathological roles in both sexes and across the lifespan. Many research groups have replicated the beneficial roles of 17β-estradiol (E2), the most potent estrogen, in memory consolidation in the past decades, even though some mechanisms are still unclear. The rapid effects of E2 in memory formation are attributed to its binding to different estrogen receptors (ER), notably the intracellular receptors ERα and ERβ, as well as the membrane ER called G protein-coupled estrogen receptor (GPER). Previous work from our laboratory demonstrated that acute post-training infusion of E2 into the dorsal hippocampus (DH) of ovariectomized female mice enhances object recognition and spatial memory consolidation via activation of ERα and ERβ, and downstream ERK signaling (Boulware *et al*., 2013). Although E2 has similarly beneficial effects on memory consolidation in male mice, these effects do not depend on ERK signaling (Koss *et al*., 2018), suggesting sex differences in the molecular mechanisms through which E2 consolidates object memories. We have also shown that post-training DH infusion of the GPER agonist G-1 enhanced memory consolidation in ovariectomized (OVX) female mice in a manner dependent on JNK/ATF2 signaling and actin polymerization (Kim *et al*., 2016, 2019). Our aim in the present study was to assess the effects of bilateral DH infusion of G-1 or the GPER antagonist G15 on object recognition and spatial memory consolidation in gonadectomized (GDX) male mice. We found that immediate post-training bilateral DH infusion of G-1 enhanced memory consolidation in object placement and object recognition tasks, as previously demonstrated in OVX female mice. As in females, treatment of GDX males with G-15 impaired memory consolidation in both tasks. Interestingly, GPER activation in the DH of male GDX mice did not increase the levels of phospho-JNK or

phosphor-cofilin as previously observed in female OVX mice, suggesting involvement of different signaling proteins in the effects of GPER in males. Levels of phospho-cAMP-responsive element binding protein (CREB) were elevated in the DH 30 minutes following G-1 infusion, indicating that GPER in males activates an as yet unknown mechanism that triggers CREB-mediated gene transcription. Our findings show for the first time the existence of sex differences mediating the molecular mechanisms through which GPER regulates memory. Thus, this work may open new avenues for sex-specific treatment of memory-related disorders.

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
CAMK	Calmodulin kinase
ERK	Extracellular signal-regulated kinases
ER	Estrogen receptor
JNK	C-Jun-kinase
CREB	cAMP-responsive element binding protein
DH	Dorsal hippocampus
DMSO	Dimethyl sulfoxide
E2	Estradiol
GDX	Gonadectomized
GPCR	G-coupled protein receptor
GPER	G-coupled protein estrogen receptor
MAPK	Ras/mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase
PI3K	Phosphoinositide 3-kinases
PKA	Protein kinase A
PKC	Protein kinase C
OP	Object placement
OR	Object recognition
OVX	Ovariectomized

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INTRODUCTION

Advances made by scientific research, medicine, and technology in the last few decades have not only increased people's lifespans but have also imposed the challenges of an aging society. Reports from the U.S. Census Bureau (2017) show that the number of Americans aged 65 and older is projected to increase from 52 million in 2018 to 95 million by 2060, and this rise will lead to a predictable increase in the incidence of age-related conditions such as dementia, cancer, and cardiovascular diseases. Alzheimer's disease (AD) is the most prevalent dementia, and 90% of cases have no obvious genetic or environmental cause; aging is the leading risk factor for developing this disorder that has no cure (Liang *et al*., 2021). Through accelerated approval pathways, the U.S. Food and Drug Administration (FDA) has recently authorized the prescription of two monoclonal antibodies against the misfolded protein beta-amyloid for treating AD. However, doubts have been raised about the efficacy of these drugs and medical societies are still evaluating the actual benefit of prescribing them, which emphasizes the need for the development of therapies targeting different pathophysiological mechanisms underlying the development and progression of this neurodegenerative disorder (Brockmann *et al*., 2023; Gandy & Ejrlich, 2023; Valiukas *et al*., 2022).

Memory impairment is one of the most debilitating symptoms of AD, and estrogens are associated with memory enhancement in hundreds of pre-clinical studies (Taxier *et al*., 2022; Gross *et al*., 2022, Fleischer *et al*., 2021). However, the translation of pre-clinical findings into clinical benefits for cognitive function in women has been complicated by a variety of factors, including type of estrogen treatment used, cardiovascular health, age, and duration between menopause onset and start of treatment. For example, the large randomized the Women Health

Initiative (WHI) trial of postmenopausal women over age 65 taking conjugated equine estrogens with or without medroxyprogesterone acetate reported reductions in hip fractures and colon cancer, but increased incidence of cardiovascular disease and breast cancer (WHI Study Group, 1998). However, this trial has been heavily criticized for the advanced age and generally poor cardiovascular health of the participants, as well as the long delay between menopause onset and treatment, and subsequent studies have demonstrated greater benefit of estradiol-based treatments for relief of menopausal symptoms in healthier women in their 50s (Miller *et al.*, 2021). Although estrogen use does not appear to improve cognition in women with established AD (Manson *et al.*, 2013; Wysowski *et al.*, 1995), estrogen use is associated with reduced AD risk and E2 treatment early in the menopausal transition is associated with reduced amyloid deposition, particularly in women at highest risk of AD (Zandi *et al.*, 2002). Taken together, the findings from clinical trials urge researchers in the field to look for alternative pathways that could result in translating the positive effects of estrogens observed in preclinical studies into safe and efficient therapeutical options for women living in the menopause therapies (Chen *et al.*, 2022). At this point, a better understanding of the different types of estrogen receptors may play an important role. As our laboratory has previously demonstrated, the agonism of GPER is associated with enhanced object and spatial memory consolidation in ovariectomized female mice tested in hippocampus-dependent object recognition and object placement tasks, respectively (Kim *et al.*, 2019; 2016). This benefit did not depend on the same intracellular signaling pathways activated by E2, suggesting that it is possible to obtain the benefits of estrogens on memory through different cellular mechanisms (Kim *et al.*, 2019; 2016).

Estrogens and memory

Estrogens are cholesterol-derived hormones. They have been traditionally associated with their roles in reproduction, but in the past three decades, their influence in myriad functions in the central nervous system (CNS) has been elucidated, expanding our comprehension of estrogens in essential cognitive functions, such as learning and memory (Taxier *et al*., 2020). For example, breakthrough studies in the field published in the early 1990s described how fluctuations in circulating levels of E2, the most potent circulating estrogen, are associated with dynamic changes in dendritic spine density on pyramidal neurons in the CA1 region of the hippocampus – a brain region situated in the medial temporal cortex and necessary for spatial and recognition memory consolidation (Rocks & Kundakovic, 2022; Woolley *et al*., 1997; Gould *et al*., 1990). Interestingly, early studies also demonstrated how the exogenous administration of E2 in gonadectomized female mice increased the density of dendritic spines in this same region (Woolley & McEwen, 1994).

The sensitivity of the CA1 region of the hippocampus can be explained by the massive presence of all three primary ERs in those areas (Milner *et al*., 2001; Milner *et al*., 2005; Akama *et al*., 2013). Estrogens exert their effects in the CNS through at least three main types of ERs: ER alpha (ERα), ER beta (ERβ), and G-protein coupled ER (GPER). Their localization within cellular compartments is still a matter of debate, but ERα and ERβ can be found in the plasma membrane, cytosol, and in the nucleus, whereas the GPER is a transmembrane receptor also localized to the endoplasmic reticulum (Prossnitz *et al*., 2008; Kumar & Chambon, 1988). The mechanisms of action for steroid receptors can be divided into classical (or genomic) or non-classical (or non-genomic). In the classical mechanism, lipid-derived ligands (such as estrogens) cross the bilipid layer membrane of the cells and bind to ERs situated in the cytosol, thereby causing the formation

of homo or heterodimers that then bind to estrogen response elements in the DNA, leading to gene transcription (Frick, 2015).

However, although this classical mechanism explains many slow and long-term effects of estrogen signaling, it is insufficient to explain the rapid effects mediated by estrogen in the cells. Some of the rapid effects modulated by estrogens in neurons, for example, can be understood by the non-classical activation of ERs, such as the crosstalk of ERs with metabotropic glutamate receptors (mGluRs) and tyrosine kinase receptors, which leads to the phosphorylation of protein kinases (MEK/ERK, PI3K/Akt, PKA, PKC, JNK) and subsequent increases in protein synthesis and gene expression, and modification of ion channels and cytoskeleton dynamics (Frick & Kim, 2018; Boulware *et al*., 2013).

The role of GPER in memory consolidation

After being described in the early 90s as GPR30, an orphan G-protein coupled receptor (GPCR), the membrane estrogen receptor GPER would receive its name only in 2007 by the International Union of Basic and Clinical Pharmacology (IUPHAR) (Arterburn & Prossnitz, 2023; Nilsson *et al*., 2011). GPER is a 7-transmembrane GPCR located in the plasma membrane, the endoplasmic reticulum, and the Golgi apparatus (Barton *et al*., 2018). The activation of GPER by E2 or G-1 (a high-affinity agonist) activates intracellular signaling pathways through cascades involving Gαs and Gαi/o, leading to an increase in cyclic adenosine monophosphate and phosphoinositide 3-kinase or Src protein kinase, respectively (Filardo *et al*., 2000). Activation of GPER is also associated with phospholipase C and changes in intracellular receptors involved in the cytosolic balance of calcium, such as the inositol receptor and ryanodine receptor (Filardo *et al*., 2007). This kinase activity leads to alterations in gene expression, for instance, c-fos, cyclin A

and D1, connective tissue growth factor, fatty acid synthase, and vascular endothelial growth function (Prossnitz & Arterburn, 2015).

Moreover, the stimulation of the PI3K pathway by GPER, leading to increased production of phosphatidylinositol 3,4,5, was associated with the activation of the transcription factor SF-1, resulting in aromatase Cyp19a1 expression and, consequently, increased E2 synthesis (Prossnitz & Arterburn, 2015). The activation of GPER in ovariectomized females mice also leads to a significant increase in the levels of PSD-95, which correlates with excitatory synapse levels in the CA3 region of the hippocampus (stratum radiatum, stratum lucidum, and stratum oriens); a similar finding was observed with E2 administration. Interestingly, however, the same study demonstrated that the GPER-induced increase in PSD-95 levels was not driven by phosphorylation of Akt as observed after E2 administration (Waters *et al*., 2015; Spencer-Segal *et al*., 2012). These findings suggest important and unique features associated with GPER activation at the synaptic level.

In the CNS, GPER is expressed in the prefrontal cortex, hippocampus, hypothalamus, pituitary, midbrain, cerebellum, spinal cord, and the dorsal root ganglia of both female and male rodents, and the possible benefits of its agonism for learning and memory has been consistently replicated across laboratories (de Souza *et al*., 2021; Kim *et al*., 2019; Machado *et al*., 2018; Prossnitz & Hathaway, 2015; Brailoui *et al*., 2007). GPER expression in the hippocampus and amygdala in rats differs significantly between the sexes and across the estrus cycle. For example, expression of immunoreactive GPER cells was higher in males than in diestrus females in the CA1-CA3 regions of the hippocampus and in the molecular layers of the dentate gyrus, as well as higher in estrus females than in diestrus females in the *strata oriens and radiatum-lacunosum-moleculare* of CA1-CA3 and in the molecular layer of the dentate gyrus (Llorente *et al*., 2020). In the hippocampus, GPER facilitates synaptic plasticity, as the GPER agonist G-1 increased

excitatory postsynaptic potentials (EPSPs) in hippocampal slices from ovariectomized ERα knockout and ERβ knockout mice (Kumar *et al*., 2015) and increased both miniature excitatory synaptic current (mEPSC) frequency and amplitude in females (Oberlander and Woolley, 2016).

Accordingly, GPER activation has been associated with memory facilitation. In the earliest studies to examine its effects on learning and memory, Hammond *et al*. (2009, 2011) demonstrated that chronic administration of G-1 to ovariectomized rats was associated with enhanced cholinergic function in the hippocampus and improved spatial working memory in a delayed matching-to-position T-maze task. In contrast, they found that systemic antagonism of GPER using the compound G-15 impaired memory in this task among female rodents (Hammond *et al*., 2012). After subcutaneous administration of G-1, ovariectomized young female rats demonstrated better performance in hippocampal dependent-tasks such as object recognition and object placement, as well as in social recognition paradigms; these effects were associated with increased density of CA1 dendritic spines 40 min after drug administration (Gabor *et al*., 2015).

More recently, our lab has used targeted intracranial infusions to show a key role for GPER in mediating memory consolidation in ovariectomized female mice. In short, we found that immediate post-training bilateral DH infusion of G-1 enhances memory in the OR and OP tasks, whereas G-15 impairs memory consolidation (Kim *et al*., 2016, 2019). G-15 blocked the memory-enhancing effects of G-1, supporting this as a GPER-mediated effect. Interestingly, although immediate post-training systemic administration of G-1 in male adult rats improved their memory in OR, drug infusion 3 or 6 hours post-training failed to produce the same improvements, suggesting a role for GPER in the early stages of memory consolidation (de Souza *et al*., 2021).

The mechanisms underlying GPER-induced memory facilitation in female mice involve rapid activation of c-jun N-terminal kinase (JNK) signaling in the DH, leading to actin polymerization and increased CA1 dendritic spine density (Kim *et al*., 2016, 2019). Interestingly, the cell signaling mechanisms through which GPER influences memory and spine density differs from that of E2 in females. In ovariectomized female mice, phosphorylation of p42ERK in the DH is necessary for E2 to increase CA1 dendritic spine density and enhance object recognition or spatial memory consolidation (Tuscher *et al*., 2016; Fernandez *et al*., 2008). The effects of E2 on ERK and memory in females are mediated by ERα and ERβ, as ERK inhibition prevents agonists of each receptor from enhancing object recognition or spatial memory consolidation (Boulware e*t al*., 2013). In contrast, however, G-1 does not increase p42ERK phosphorylation, nor does inhibition of ERK activation prevent G-1 from enhancing memory in female mice (Kim *et al*., 2016). Moreover, E2 does not activate JNK signaling in the DH, nor does G-15 or a JNK inhibitor prevent E2 from activating p42ERK or enhancing increasing object recognition or spatial memory consolidation (Kim *et al*., 2016). Thus, in female mice, GPER in the DH appears to regulate memory and spine density via different signaling mechanisms than ERα and ERβ.

The implications of GPER in synaptic plasticity were also confirmed by electrophysiological studies demonstrating that G-1 increased excitatory postsynaptic potentials (EPSPs) in 8 hippocampal slices from ovariectomized ERα knockout and ERβ knockout female mice (Kumar *et al*., 2015). In addition, Oberlander and Woolley (2016) used whole-cell voltage clamp to show that G-1 increased both miniature excitatory synaptic currents (mEPSCs) frequency and amplitude in females, but not males, pointing towards sex differences in hippocampal glutamatergic synaptic transmission associated with GPER activation.

Finally, in male rodents, little is known about the importance of GPER for memory. As described earlier, systemic administration of G-1 in intact male rats was associated with better spatial memory in the OP and inhibitory avoidance tasks when given immediately after training, but not 3 or 6 hours after training, suggesting that GPER plays an important role in the initial stages of memory consolidation (de Souza *et al*., 2021). Systemic administration of the GPER antagonist G-15 impaired memory in both tasks (de Souza *et al*., 2021). Although the behavioral tasks used in these experiments depend on hippocampal function, it is important to note that systemic injection of the compounds could simultaneously activate GPER expressed in other brain regions. Thus, the extent to which hippocampal GPER is involved in mediating memory in males is unclear. It is also important to note that same behavioral findings may derive from different intracellular mechanisms, given our laboratory's previous reports showing sex differences in the memory-enhancing effects of E2 in OR and OP that depend on p42ERK phosphorylation in females but not males (Koss *et al*., 2018). Although it is unclear what signaling mechanisms mediate E2-induced memory enhancement in males, E2 did increase phosphorylation of the transcription factor CREB in both sexes (Koss *et al*., 2018).

Role of estrogens in learning and memory in male rodents

Although our lab has published extensively on the cellular mechanisms through which E2 regulates memory consolidation and hippocampal function and structure, most of these studies have focused on the role of E2 in female mice only (Koss & Frick, 2017). ERα, ERβ, and GPER are also widely expressed in hippocampal neurons and astrocytes in males, and the levels of E2 are also high in this region; some studies focusing on steroidogenesis in the hippocampus of male rodents demonstrated that levels of the enzyme aromatase - involved in the last step of E2 synthesis - are higher in males than in females at low estrogen points of the estrous cycle (Hojo *et al*., 2009;

Brailoui *et al.*, 2007; Li *et al.*, 1997; Loy *et al.*, 1988). These data suggest a potentially important role for estrogens in mediating learning and memory in males, but it is unclear how this might overlap with previous findings in females and whether similar cell-signaling mechanisms underlie memory effects in males and females.

E2 has been shown to regulate hippocampal structure and function in males. E2 increases CA1 spinogenesis in studies using hippocampal slices from gonadally-intact male mice, and ERα predominantly mediated this effect (Hasegawa *et al.*, 2015; Hojo *et al.*, 2015). This effect is also dependent on activation of kinases such as PKA, protein kinase C, PI3K, ERK, CaMKII, LIM kinase, and calcineurin, and the pharmacological inhibition of some of these intracellular signaling kinases prevented E2 from enhancing long-term potentiation in these slices (Hasegawa *et al.*, 2015; Hojo *et al.*, 2015). Interestingly, the positive effects of E2 in NMDA-dependent LTP rely on the effects of ERβ; ERβ also seems to mediate postsynaptic sensibility to glutamate, whereas ERα regulates the presynaptic release of this neurotransmitter (Oberlander & Woolley, 2016). More recently, E2 was also shown to be necessary for synaptic potentiation in ovariectomized female rats, but not for gonadectomized male rats; interestingly, synaptic potentiation in females was more dependent on calcium permeable AMPA receptors conductance than in males suggesting a possible contributor to sex differences in potentiation (Jain & Woolley, 2023).

Regarding the effects of E2 on learning and memory in male mice, pre-training and post-training administration of E2 is associated, in general, with the enhancement in spatial and recognition memory tasks, but the mechanisms are poorly understood. Gibbs and Johnson (2008) used a 12- arm radial maze task for assessing working and reference memory and investigated the differences between male and female rats in the execution of this task. They found that male rats performed significantly better than females on the working memory component of the task but not

on the reference component. This advantage disappeared when males were gonadectomized. In males, chronic testosterone administration was associated with poor accuracy and reference memory performance (Gibbs & Johnson, 2008). In contrast, the chronic administration of E2 restored working memory in ovariectomized females (Gibbs & Johnson, 2008). E2 was also able to restore spatial working memory in gonadectomized male rats (Locklear & Kritzer, 2014). Our laboratory has investigated the role of E2 in mediating memory consolidation in males, and we have found exciting findings related to sex and gonadal status (Koss *et al*., 2018). A bilateral acute post-training DH infusion of E2 enhanced the memory of gonadally-intact male and gonadectomized male mice in OP and OR – which is a consistent effect reported in female mice ovariectomized mice. Interestingly, the DH infusion of E2 was not associated with increased ERK or Akt phosphorylation in the DH. The pharmacological inhibition of ERK activation in the DH failed to prevent the memory-enhancing effects of E2 in males. In addition, we observed an increase in the phosphorylation of the CREB following DH E2 infusion in males. These findings suggest that DH E2 infusion mediates memory enhancement in both sexes but through different mechanisms than in females (Koss *et al*., 2018). Other work from our laboratory also showed that the manipulation of the steroidogenesis process in the brain, through the infusion of letrozole (an aromatase inhibitor), impaired OP and OR memory consolidation in gonadectomized, but not intact, male mice, and the blockade of androgen receptors dose-dependently impaired memory consolidation among gonadally-intact male mice in these tasks (Koss *et al*., 2019).

Collectively, considerable evidence demonstrates a critical role of E2 in regulating memory consolidation through mechanisms as kinase phosphorylation, dendritic spine density, and synaptic plasticity in both sexes (Fernandez *et al*., 2008; Hasegawa *et al*., 2015; Tuscher *et al*., 2016b). The memory-enhancement observed from the activation of GPER has been studied

predominantly in females, and these studies have shown that GPER agonism is associated with unique intracellular signaling pathways activations (e.g., JNK phosphorylation) (Kim *et al*., 2016, 2019). In males, there is a lack of data regarding the role of this receptor in memory consolidation, although it seems that the pharmacological agonism of GPER results in memory enhancement in hippocampal-dependent memory tasks, as described for females, although localization of this effect to the hippocampus and the nature of the intracellular signaling events involved remains unexplored (de Souza *et al*., 2021). Therefore, the primary goal of this work was to determine the extent to which dorsal hippocampal GPER regulates object recognition and spatial memory consolidation in gonadectomized male mice and to assess whether JNK signaling plays a pivotal role as observed in ovariectomized mice (Kim *et al*., 2016, 2019).

METHODS

Subjects

Male C57BL/6 mice (n = 10-12/group) were obtained from Taconic Biosciences (Germantown, NY) at 8 weeks of age and housed individually in shoebox cages in a room (22-23°C) with a 12/12-h light-dark cycle. Food and water were provided *ad libitum*. Mice were handled for 5 min/day for three days before the start of behavioral testing to get accustomed to the experimenter. All procedures were conducted from 10:00 to 17:00 h in a quiet room, and experimenters conducting behavioral testing were blinded to treatment regimen. Mice were monitored regularly throughout the experiments for any sign of pain or distress. All experimental procedures were approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Animals.

General Experimental Design Overview

Male mice first underwent a dual surgical procedure in which they were bilaterally

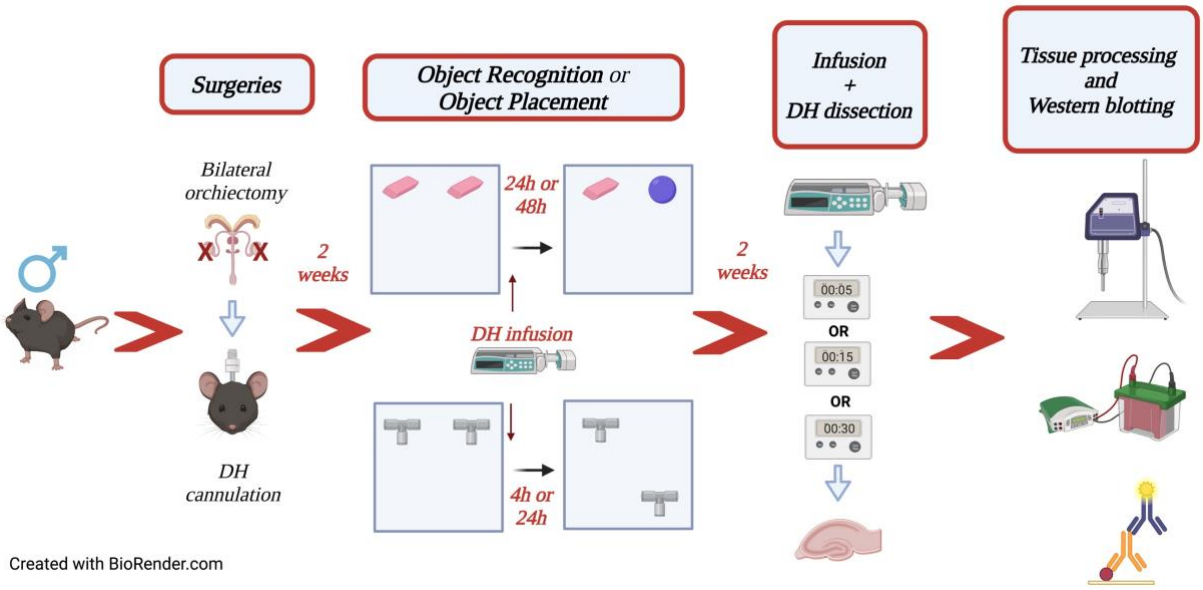

Figure 1. Schematic illustrating the general experimental design. Young male C57BL/6 mice (6-8 weeks old) were gonadectomized, cannulated, and given 7-14 days of recovery before behavioral testing. All mice were trained in OR and OP, immediately after which they received DH infusion of vehicle (4% or 16% DMSO), G-1 (4 or 8 ng/hemisphere), or G-15 (1.85 or 7.4 ng/hemisphere). The interval between training and testing depended on the drug and task. Fourteen days after the final behavioral test, mice were infused and DH tissue collected 5, 15, or 30 min later for Western blotting.

gonadectomized and then were bilaterally implanted with cannulae aimed at each DH (**Fig. 1**). Mice were allowed seven days to recover before the start of behavioral habituation. Immediately after training, mice were infused with vehicle or G-1 (to test effects of GPER agonism) or vehicle or G-15 (to test effects of GPER antagonism). For G-1, memory consolidation was evaluated 24 h later in OP and 48 h later in OR. For G-15, memory was tested 4 and 24 hours later in OP and OR, respectively. A minimum of 14 days separated OR and OP testing, the order of which was counterbalanced within a group; this interval allowed metabolic clearance of the drugs from the brain and for any acute neural effects of infusion to dissipate prior to the next infusion. Finally,

ten days after the final behavioral test, mice were infused again and the DH was collected bilaterally 5, 15, or 30 min later for Western blotting.

Surgical Procedures

At least four days after arrival in the laboratory, mice underwent bilateral orchiectomy immediately followed by bilateral implantation of chronic indwelling stainless-steel guide cannulae into the DH as described previously (Koss *et al*., 2018). Briefly, mice were anesthetized with 5% isoflurane in 100% oxygen for induction and secured in a stereotaxic apparatus (Kopf Instruments). Anesthesia was maintained at 2-3% isoflurane throughout surgery and analgesia was provided via a 5 mg/kg subcutaneous injection of Rimadyl prior to surgery. For gonadectomy (GDX) surgeries, a midline incision was made on the scrotal sac, and then the testes were isolated and carefully separated from the fat, tied off at the vas deferens, and removed. The incision was closed with monofilament sutures. For cannulations, mice were implanted with bilateral guide cannulae (22 gauge; C232G, Plastics One Inc.) aimed at each hemisphere of the DH (1.7 mm AP, ±1.5 mm ML, 2.3 mm DV). Dummy cannulae (C232DC, Plastics One Inc.) were inserted into each guide cannula to maintain patency. Cannulae were fixed to the skull with dental cement (Darby Dental Supply), which also served to close the wound. During post-operative recovery, mice were carefully observed for any signal of discomfort and received ¼ of a 2 mg Rimadyl tablet on the first post-operative day, and as then needed.

Drugs and Infusions

Post-training drug infusions were performed by gently restraining mice to remove the dummy cannulae, followed by placement of an infusion cannula into each guide cannula (C3131; DH; 28 gauge, extending 0.8 mm beyond the 1.5 mm guide). The infusion cannula was attached to PE50 polyethylene tubing attached to a 10 µl Hamilton syringe. Infusions were controlled by a

micro infusion pump (KDS Legato 180, KD Scientific) at a rate of 0.5 µl/min for 1 min. Infusion cannulae remained in place for one minute after each infusion to avoid diffusion of drugs back up the cannula track.

The selective GPER agonist G-1 (1-[4-(6-bromobenzo[1,3]dioxol-5yl)-3a,4,5,9b-tetrahydro3Hcyclopenta [c]quinolin-8-yl]-ethanone; Azano Biotech) was dissolved in 16% dimethylsulfoxide (DMSO) and infused at doses of 4 or 8 ng/hemisphere into the DH as per the lab's previous work (Kim *et al*., 2016; 2019). The GPER-selective antagonist G-15 ((3aS*,4R*, 9bR*)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c] quinolone; Azano Biotech) was dissolved in 2% DMSO and infused at doses of 1.85 or 7.4 ng/hemisphere as described previously (Kim *et al*., 2016; 2019). Vehicle controls for G-1 and G-15 were 16% and 2% DMSO, respectively.

Memory Assessment

The effects of G-1 and G-15 on memory consolidation were examined using the OP and OR tasks, which assess spatial and object recognition memory, respectively (Fernandez *et al*., 2008; Boulware *et al*., 2013; Tuscher *et al*., 2016) (**Fig. 2**). OP and OR were conducted in a white open field box (width, 60 cm; length, 60 cm; height, 47 cm). Before behavioral training, mice were handled for 1 min/day for three days. On the second handling day, a single Lego Duplo block was placed in the home cage to acclimate mice to objects. Following handling, mice habituated to the

empty open field box for 5 min/day for two days. During habituation, mice could move freely in the apparatus without objects.

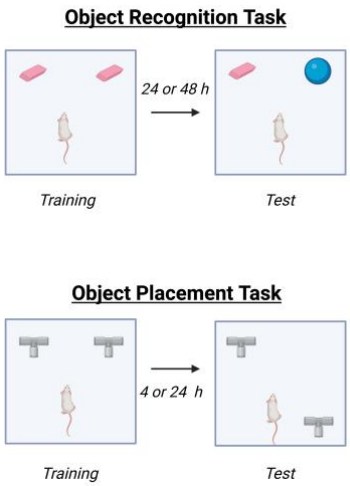

Figure 2. Schematic of object recognition (OR) and object placement tasks (OP). See text for description.

Following habituation, mice underwent OP and OR training, during which mice were given up to 20 min to accumulate 30 s exploring two identical objects placed in the upper right and left corners of the open field box. Experimenters manually scored in real-time the amount of object exploration using ANYmaze tracking software (Stoelting). Object exploration was counted when the mouse's nose and/or front paws are directed towards and/or touching the objects. Different objects were used for OP and OR, and all objects used were counterbalanced across mice to account for any potential object preferences. Immediately following training, mice were given bilateral DH infusions of vehicle, G-1, or G15 as described above. These treatments were administered post-training to pinpoint effects of G-1 and G-15 specifically on the consolidation phase of spatial and object recognition memory formation. The mice that did not accumulate 30 s of exploration during training were re-trained 4-7 days later with different objects.

The interval between training and testing varied depending on the drug infused immediately after training. For treatment with G-1, mice were tested 24 h and 48 h later for OP and OR, respectively, and for treatment with G-15, mice were tested 4 h and 24 h later for OP and OR, respectively. Longer time points were used for G-1 based on previous evidence that vehicle-treated gonadally-intact male mice do not remember the location and identity of objects at these time points (Koss *et al.*, 2018), thus allowing us to observe potential memory enhancing effects of

G-1. On the other hand, gonadally intact male mice can remember object location and identity at the shorter delays (Frick and Gresack, 2003; Fortress *et al.*, 2013), permitting observation of potential memory-impairing effects of G-15. For OP testing, the least explored training object was moved to the box's lower right or left corner. For OR testing, the least-explored training object was replaced with a novel object. Mice were given 20 min to accumulate 30 s of exploration during OP and OR testing. Mice that remember the location and identity of the training objects should spend more time than chance with the moved and novel objects. Chance is designated at 15 s because this value represents an equal exploration of both objects (Frick and Gresack, 2003). If mice did not accumulate 30 s of exploration within 20 min, then they were given up to three subsequent chances to successfully do so. All mice were given two weeks between bouts of testing to ensure that any acute effects of previous drug infusions dissipated before subsequent infusion.

Western Blotting Analysis

Western blotting was performed as described previously to measure effects of G-1 on cell signaling proteins (Kim *et al.*, 2019; Taxier *et al.*, 2022). Mice were cervically dislocated and decapitated, and the DH was dissected bilaterally on an ice-cold plate 5, 15, or 30 min after infusion. The overlying parietal, occipital, and temporal cortices were removed using a scalpel and forceps to expose the DH. Horizontal cuts were made at a 45° angle through each side of the DH at the level of the base of the superior colliculus. The fornix was transected with the scalpel blade. The entire DH, including the dentate gyrus and cornu ammonis fields, were bilaterally removed with forceps and placed in a 1.5 ml microcentrifuge tube. Tissue samples were immediately weighed and frozen on dry ice, and then stored at 80°C until homogenization. DH tissues were resuspended to 50 µl/mg in lysis buffer and homogenized using a sonicator (Branson Sonifier 250) as described previously (Taxier *et al.*, 2022; Fortress *et al.*, 2015). Proteins were electrophoresed

on 10% Tris-HCl precast gels (Bio-Rad) and transferred to PVDF membranes (Bio-Rad). Western blots were blocked with 5% skim milk and incubated with primary antibodies (phospho-ERK, phospho-Akt, phospho-PI3K, phospho-JNK, phospho-cofilin, phospho-CREB 1:1000; Cell Signaling Technology) overnight at 4°C. Blots were incubated for 1 h at room temperature with a rabbit HRP-conjugated secondary antibody (1:5000; Cell Signaling Technology) and developed using West Dura chemiluminescent substrate (Pierce). A ChemiDoc MP gel imager (Bio-Rad) detected signal correlating with protein expression. Densitometry was performed using Carestream Molecular Imaging Software (Carestream Healthcare). Blots were then stripped with 0.2 M NaOH and incubated with antibodies (total-ERK, total-Akt, total-PI3K, and total-JNK, total-cofilin, total-CREB 1:1000; Cell Signaling Technology) for protein normalization. Data were represented as percent immunoreactivity relative to vehicle controls. Treatment effects were measured within single gels (n = 10-14/group).

Statistical Analyses

Statistical analyses were conducted using GraphPad Prism 9 (La Jolla, CA). For each behavioral experiment, separate one-sample t-tests were performed within each group to determine if the time spent with the novel object differs from chance (15 s; Frick and Gresack, 2003; Boulware *et al*., 2013; Tuscher *et al*., 2016; Taxier *et al*., 2022). To assess between-group treatment effects, one-way analyses of variance (ANOVAs) were conducted to assess potential main effects of Treatment, followed by Tukey's posthoc tests (Kim *et al*., 2016; Kim *et al*., 2019). The time to accumulate 30 s of exploration objects were analyzed with one-way ANOVAs. Normalized western blot data were analyzed using two-way ANOVAs with Treatment (vehicle, G-1) and Time point (5, 15, 30 min) as dependent variables. Statistical significance was determined at $p \leq 0.05$.

RESULTS

GPER in the DH Regulates Memory Consolidation in GDX Mice

We first evaluated whether immediate post-training DH infusion of G-1 (4 or 8 ng/hemisphere (ng/h)) could facilitate memory consolidation in gonadectomized male mice. Mice infused with either 4 or 8 ng G-1 spent significantly more time with the novel object than chance (4 ng/h: $t_{(12)} = 3.64$, $p = 0.003$; 8 ng/h: $t_{(12)} = 6.60$, $p < 0.0001$; **Fig. 3A**), suggesting that both doses enhance OR memory consolidation in males. The main effect of Treatment was also significant in the one-way ANOVA ($F_{(2,36)} = 12.25$, $p < 0.0001$), and posthoc tests showed that mice treated with 4 or 8 ng/hemisphere G-1 spent significantly more time with the novel object than those infused with vehicle (4 ng/h: $p = 0.002$; 8 ng/h: $p = 0.0001$; **Fig. 3A**). Elapsed time to accumulate 30 s of exploration did not differ among the groups ($F_{(2,36)} = 3.03$, $p > 0.05$; vehicle = 425.2 ± 48.07; 4 ng/h G-1 = 600.8 ± 58.55; 8 ng/h G-1 = 580.2 ± 58). In OP (**Fig. 3B**), only mice infused with 8 ng/h G-1 significantly explored the moved object more than chance ($t_{(11)} = 2.3$, $p = 0.04$). Interestingly, the 4 ng/h G-1 group spent significantly less time than chance with the moved object ($t_{(9)} = 4.08$, $p = 0.002$), suggesting memory impairment with this

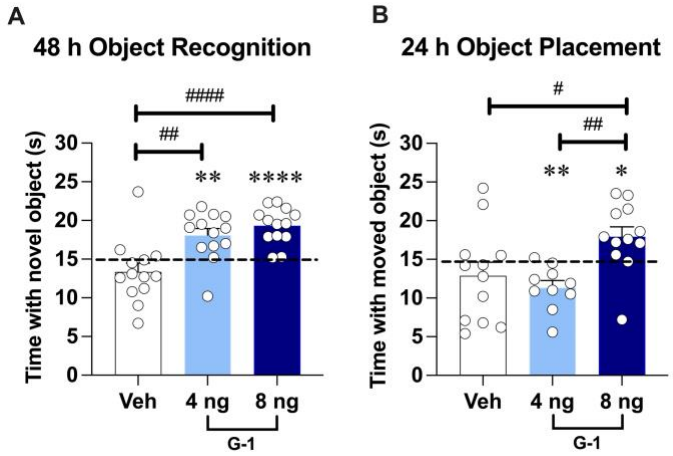

Figure 3. GPER agonism by G-1 enhances memory consolidation in GDX male mice. **A**, Mice receiving DH infusion of G-1 (4 or 8 ng/h) spent significantly more time with the novel object than chance (dashed line at 15 s). Both G-1 groups also spent significantly more time with the novel object than the vehicle group. **B**, Mice receiving DH infusion of 8 ng/h G-1 spent significantly more time than chance with the moved object, whereas those treated with vehicle or 4 ng/h G-1 did not. Each bar represents the mean ± standard error of the mean (SEM) time (s) spent with the novel (OR) or moved (OP) object. (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ relative to chance; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$ relative to vehicle).

dose. The main effect of Treatment was significant ($F_{(2,31)} = 5.99$, $p = 0.006$; **Fig. 3B**) due to significant differences between the 8 ng/h G-1 group and both the vehicle ($p = 0.03$) and 4 ng/h ($p = 0.008$) groups. As with OR, elapsed time to accumulate 30 s of exploration in OP during testing did not differ among the groups ($F_{(2,31)} = 0.98$, $p > 0.05$; vehicle = 300.8 ± 23.78; 4 ng G-1 = 350.9 ± 34.75; 8 ng G-1 = 355.4 ± 34.39). Collectively, these data suggest that GPER activation dose-dependently enhances both OR and OP memory consolidation in GDX males. Whereas 8 ng/h G-1 enhanced memory in both tasks, the effects of 4 ng/h G-1 were task-dependent.

We next evaluated the extent to which GPER antagonism impaired memory consolidation in GDX mice. OR and OP memory consolidation were tested 24 and 4 h after training, delays at which vehicle infused OVX mice show intact memory for the identity and location of the training objects (Kim *et al.*, 2016; Kim *et al.*, 2019). Accordingly, vehicle-infused GDX males spent significantly more time with the novel ($t_{(11)} = 4.36$, $p = 0.0011$; **Fig. 4A**) and moved ($t_{(11)} = 2.553$, $p = 0.0268$; **Fig. 4B**) objects than chance. In

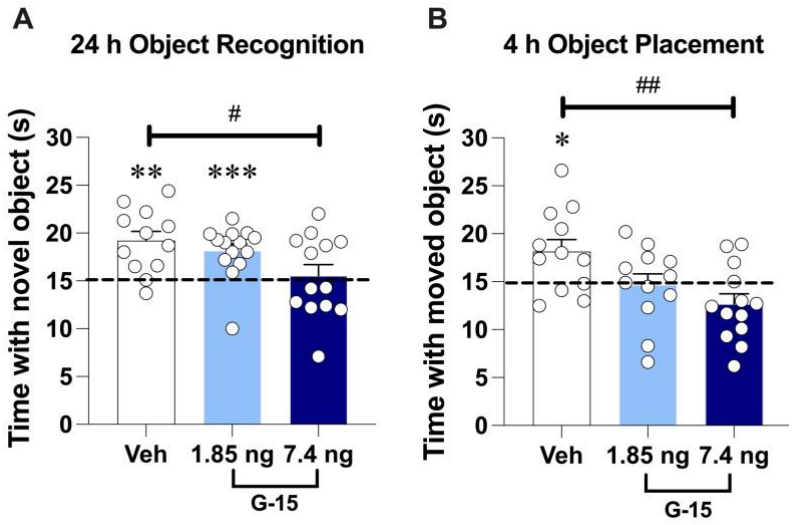

Figure 4. GPER antagonism by G-15 impairs memory consolidation. **A**, Mice receiving DH infusion of 7.4 ng/h G-15 spent significantly less time than chance (dashed line) with the novel object. This group also spent less time with the novel object than vehicle, indicating memory impairment. **B**, Mice receiving DH infusion of G-15 (1.85 ng/h or 7.4 ng/h) spent significantly less time than chance with the moved object, but only the group that received 7.4 ng/h of G-15 spent significantly less time with the moved object than vehicle. Each bar represents the mean ± standard error of the mean (SEM) time (seconds) spent with the novel (OR) or moved (OP) object. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ relative to chance; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ relative to vehicle).

contrast, mice infused with 7.4 ng/h G-15 spent chance amounts of time with the novel object ($t_{(12)}$ = 0.4481, p = 0.6621) and spent nearly significantly less time with the moved object than chance (($t_{(12)}$ = 2.16, p = 0.0517), suggesting that 7.4 ng/h G-15 impairs OR and OP memory consolidation. Interestingly, the 1.85 ng/h dose of G-15 impaired OP ($t_{(11)}$ = 0.2936, p = 0.7745) but had no detrimental effect on memory in OR ($t_{(13)}$ = 4.248, p = 0.001). These treatment effects were reflected in one-way ANOVAs, such that the main effects of Treatment were significant for both OR ($F_{(2,36)}$ = 3.704, p = 0.0345) and OP ($F_{(2,34)}$ = 5.785, p = 0.0069). Posthocs showed that the 7.4 ng/h group spent significantly less time with the novel (p = 0.0332) and moved (p = 0.0056) objects than the vehicle group. The 1.85 ng/h group did not differ from the vehicle group in either task. Elapsed time to accumulate 30 s of exploration did not differ among the groups for either OR ($F_{(2,36)}$ = 2.15, p > 0.05; vehicle = 457.2 ± 46.15; 1.85 ng G-15 = 493.9 ± 39.17; 7.4 ng G-15 = 622.2 ± 81.76) or OP ($F_{(2,34)}$ = 0.04, p > 0.05; vehicle = 475.5 ± 49.08; 1.85 ng G-15 = 464.4 ± 75.08; 7.4 ng G-15 = 453.4 ± 44.94). Together, these data indicate that G-15 dose-dependently impaired OR and OP memory consolidation in GDX mice, and that spatial memory consolidation in GDX males may be more susceptible to the memory-impairing effects of GPER antagonism.

Collectively, the results of these behavioral pharmacology studies suggest a key role for GPER in the DH during the consolidation phase of memory for object recognition and spatial memories, such that GPER activation enhances memory whereas GPER antagonism impairs memory. To understand the downstream cellular mechanisms underlying this regulation, we next evaluated the effects of DH G-1 infusion on several intracellular signaling pathways known to regulate the effects of GPER or E2 on memory consolidation in OVX mice.

GPER Activation Did Not Increase JNK or Cofilin Phosphorylation in the DH of Male Gonadectomized Mice Within 30 Minutes

We first examined the extent DH bilateral to which infusion of G-1 (8 ng/h) in GDX mice may increase phosphorylation of the 46kD and 54kD isoforms of the protein c-Jun N-terminal-Kinase (JNK) and the actin regulatory protein cofilin. In our previous studies with OVX mice, DH levels of phospho-JNK(p46), phospho-JNK(p54), and phospho-cofilin were elevated 5 min after DH G-1 infusion, and inhibitors of JNK or actin polymerization blocked the memory-enhancing effects of G-1 (Kim *et al*. 2019, 2016). Thus, we measured these three proteins here. Surprisingly, G-1 did not increase DH phosphorylation of p46 or p54 JNK at any time point (**Figure 5A,B**), as indicated by null effects of Treatment (p46: $F_{(1,59)} = 0.99$, $p = 0.32$; p54: $F_{(1,59)} = 0.88$, $p = 0.35$), Time (p46: $F_{(2,59)} = 2.86$, $p = 0.07$; p54: $F_{(2,59)} = 2.51$, $p = 0.09$), and Treatment x Time (p46: $F_{(2,59)} = 0.48$, $p = 0.62$; p54: $F_{(2,59)} = 1.47$, $p = 0.23$).

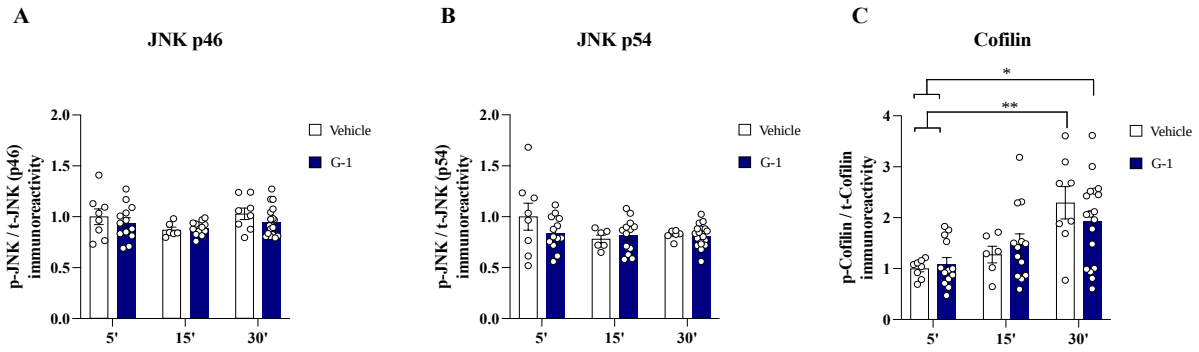

Figure 5. Effects of G-1 infusion in the JNK and cofilin signaling in the DH of GDX mice. A, B Mice receiving G-1 (8 ng/h) did not show an increase in levels of phospho-JNK p46 or p54 5, 15, or 30 minutes post-infusion. **C**, Levels of phospho-cofilin were significantly higher 30' following vehicle or G-1 infusion. Each bar represents the mean ± SEM percentage change from vehicle controls (*****$p < 0.05$, ******$p < 0.01$, relative to 5' vehicle and G-1 groups).

Analysis of cofilin phosphorylation levels (**Figure 5C**) interestingly demonstrated a significant main effect associated with Time ($F_{(2,60)} = 13.90$, $p < 0.0001$), but no main effects for Treatment ($F_{(1,60)} = 0.012$, $p = 0.91$) or Treatment x Time ($F_{(2,60)} = 0.95$, $p = 0.40$). *Post-hoc* analyses showed that the levels of phospho-cofilin in the DH of GDX male mice increased 30

minutes after infusion of either vehicle or G-1 in comparison with the 5 minute timepoint in both groups (5' Vehicle vs 30' Vehicle, p = 0.004; 5' Vehicle vs 30' G-1, p = 0.02; 5' G-1 vs 30' Vehicle, p = 0.002; 5' G-1 vs 30' G-1, p = 0.01), suggesting a non-specific effect of the infusion procedure on phospho-cofilin levels.

Together, these data indicate that GPER activation in the DH of GDX males does not rapidly trigger JNK signaling or actin polymerization as it does in OVX females, and therefore, suggest that the effects of GPER on memory consolidation in GDX males do not depend on these processes as previously demonstrated in OVX females. Thus, we next assessed activation of classical intracellular pathways associated with E2-induced memory consolidation in OVX mice.

G-1 Did Not Activate Signaling Pathways Associated with E2-Induced Memory Enhancement in GDX Mice

In OVX mice, the ability of E2 to enhance memory consolidation in the OR and OP tasks depends on rapid activation of p42 ERK (but not p44 ERK) and PI3K/Akt (Koss *et al*., 2018; Frick, 2015; Fortress *et al*., 2013; Fernandez *et al*., 2008). However, G-1 in OVX mice does not activate these signaling pathways (Kim *et al*., 2016), suggesting that the memory-enhancing effects of E2 and GPER involve different signaling pathways in OVX females. Nevertheless, it remained possible that these pathways could be involved in the effects of G-1 in males. Thus, levels of phosphorylated p42 ERK, p44 ERK, PI3K, and Akt were measured in the DH of the vehicle- and G-1-infused mice assessed for JNK and cofilin. As in OVX mice (Kim *et al*., 2016), G-1 had no effects on ERK phosphorylation at any timepoint, as illustrated by null effects of Treatment (p42 ERK: $F_{(1,61)} = 1.24$, $p = 0.27$; p44 ERK: $F_{(1,61)} = 0.08$, $p = 0.78$), Time (p42 ERK: $F_{(2,61)} = 0.50$, $p = 0.60$; p44 ERK: $F_{(2,61)} = 2.00$, $p = 0.14$), and Treatment x Time (p42 ERK: $F_{(2,61)} = 1.89$, $p = 0.16$; p44 ERK: $F_{(2,61)} = 2.37$, $p = 0.10$) (**Figure 6A, B)**. Also similar to OVX mice, G-1 did not

affect levels of phospho-PI3K (Treatment: $F_{(1,59)} = 0.03$, $p = 0.87$; Time: $F_{(2,59)} = 0.20$, $p = 0.81$; Treatment x Time: $F_{(2,59)} = 0.03$, $p = 0.96$) or phospho-AKT (Treatment: $F_{(1,47)} = 1.54$, $p = 0.22$; Time: $F_{(2,47)} = 1.12$, $p = 0.34$; Treatment x Time: $F_{(2,47)} = 1.94$, $p = 0.15$) (**Figure 6C, D)**. These

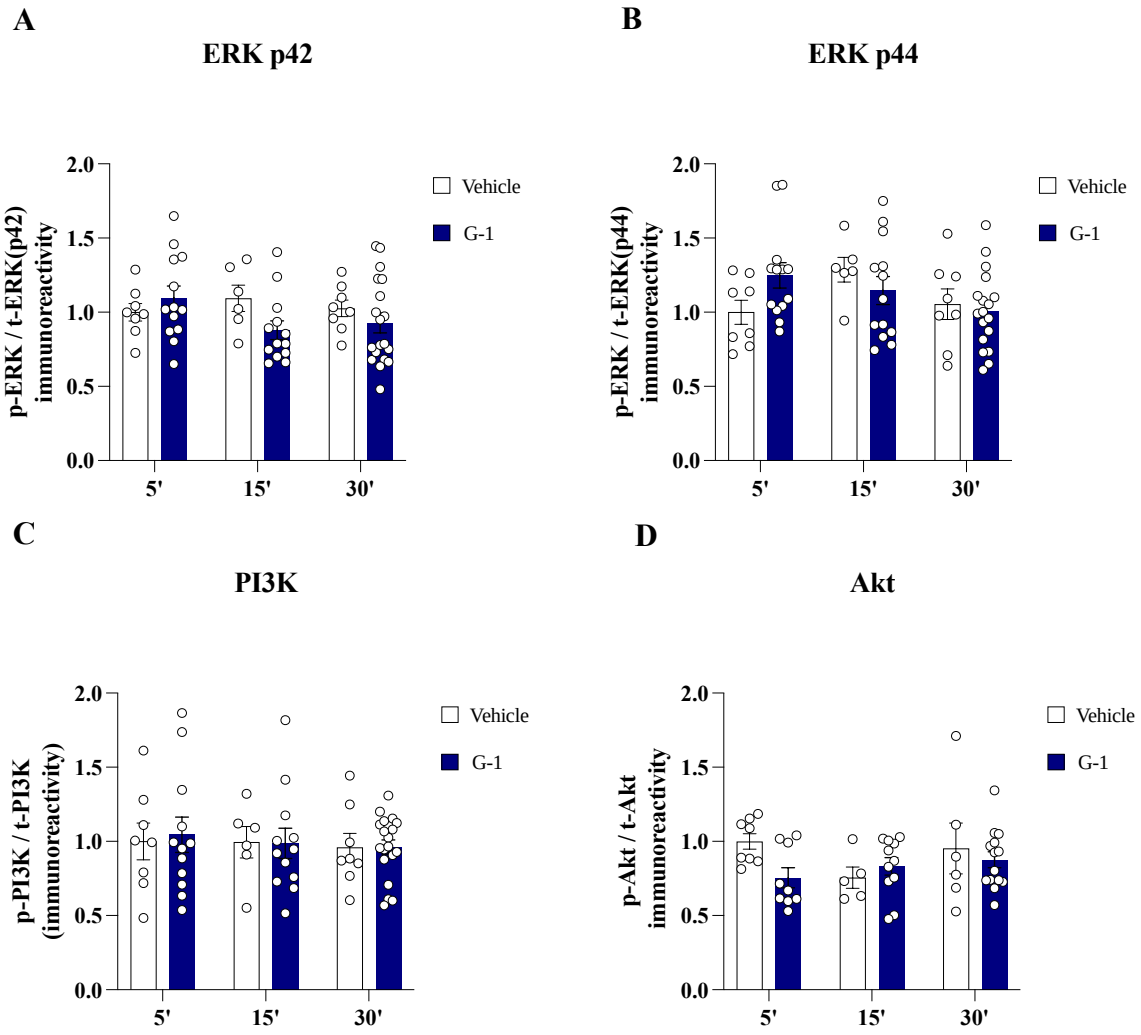

Figure 6. G-1 infusion in GDX mice did not activate ERK/PI3K/Akt pathways A, B Infusion of G-1 (8 ng/h) did not elicit significant changes in the levels of both isoforms of ERK (p42/p44) relative to vehicle. ***C, D*** GDX mice treated t with G-1 did not have significant changes in PI3K and Akt phosphorylation relative to vehicle 5-, 15-, or 30-min following infusion. Each bar represents the mean ± SEM (*$p < 0.05$).

findings suggest that the effects of G-1 on memory in GDX males do not involve ERK or PI3K/Akt signaling.

DH Infusion of G-1 in GDX Mice Increases DH CREB phosphorylation

The c-AMP response element-binding protein (CREB) has been widely studied as fundamental transcription factor for activation of required gene expression for the formation of long-term memory and increased levels of phospho-CREB are related to synaptic plasticity (Koss *et al.*, 2018 Barco, Pittenger & Kandel, 2003; Bernabeu *et al.*, 1997; Bevilaqua *et al.*, 1997). Systemic administration of G-1 in OVX rats increased phospho-CREB levels after 3 h (Machado *et al.*, 2019), and DH infusion of E2 increased DH phospho-CREB levels in OVX mice, GDX mice, and gonadally-intact male mice (Koss *et al.*, 2018). Thus, we next examined CREB phosphorylation. G-1 significantly affected phospho-CREB levels in a time-dependent manner, as illustrated by main effects of Treatment ($F_{(1,57)} = 5.08$, $p = 0.03$) and Time ($F_{(2,57)} = 2.77$, $p = 0.07$), although the Treatment x Time interaction ($F_{(2,57)} = 2.77$, $p = 0.07$) was not significant (**Fig. 7**). Tukey's *post-hoc* tests demonstrated that levels of phospho-CREB were significantly elevated 30 minutes after G-1 (8 ng/h) infusion in GDX male mice (vehicle 5' versus G-1 30', $p < 0.0001$; G-1 5' versus G-1 30', $p < 0.0001$; vehicle 15' versus G-1 30', $p <$

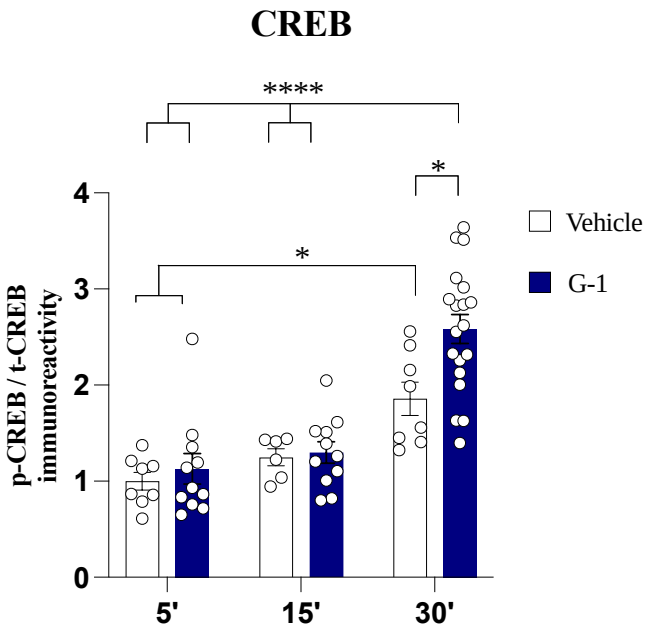

Figure 7. G-1 increases phospho-CREB levels in the DH 30 min after infusion. DH G-1 (8 ng/h) infusion significantly increases CREB phosphorylation in the DH of GDX male mice 30 min following administration. Each bar represents the mean ± standard error of the mean (SEM) (*****$p < 0.05$, ******$p < 0.01$, *******$p < 0.001$).

0.0001; vehicle 30' versus G-1 30', p = 0.01). Interestingly, the vehicle group also showed significant elevated levels of phospho-CREB 30 minutes following infusion, although the G-1 induced increase at this time point was significantly elevated relative to vehicle (Vehicle 5' versus Vehicle 30', p = 0.01 and G-1 5' versus Vehicle 30', p = 0.03, Vehicle 30' versus G-1 30', p = 0.01) (**Fig. 7)**.

DISCUSSION

The molecular and cellular mechanisms through which estrogens regulate memory remain unclear, but substantial progress has been made in recent years to pinpoint key molecules and neural processes underlying estrogenic memory modulation in the brains of females. Considerably less work has focused on males, despite the presence of E2, estrogen receptors, and *de novo* estrogen synthesis in the male brain (Koss *et al*., 2019; Frick, Kim & Koss, 2018; Luine, Serrano & Frankfurt, 2018). As such, the goal of this thesis was to better understand estrogenic regulation of memory in males. Specifically, this study focused on the membrane estrogen receptor GPER, whose activation in OVX females enhances memory by rapidly increasing JNK signaling, actin polymerization, and CA1 dendritic spine density in the DH (Kim *et al*., 2016; Kim *et al*., 2019). The present findings suggest that pharmacological activation of dorsal hippocampal GPER by G-1 also enhances object recognition and spatial memory consolidation in GDX male mice. As in females, antagonism of GPER by G-15 impaired the consolidation of both types of memory. G-1 and G-15 were administered into the DH, suggesting that these effects are specifically associated with pharmacological manipulation of GPER expressed in the dorsal hippocampus region. However, it is unclear what neural mechanisms regulate the effects of GPER in males. Unlike in females (Kim *et al*., 2019, 2016), DH infusion of G-1 did not enhance the phosphorylation of JNK or cofilin, nor did it activate the ERK or PI3K/Akt pathways. G-1 did increase the levels of

phospho-CREB in the DH 30 minutes after infusion, indicating a downstream role on gene transcription, but the signaling events leading to these effects remain unclear at the present time. These new findings indicate that GPER activation is important for object recognition and spatial memory consolidation in male GDX mice. However, the intracellular cascades involved are distinct from those described previously in female OVX mice (Kim *et al*., 2016, 2019). Our data corroborate the importance of studies investigating sex differences in memory, reflecting the epidemiology of many neurological disorders, such as Alzheimer's disease.

Effects of Post-training GPER Activation on Spatial and Object Recognition Memory Consolidation

We first demonstrated that the bilateral DH infusion of G-1 immediately after training enhanced object recognition and spatial memory consolidation in GDX male mice in a manner dependent on dose and task. The finding that GPER activation can facilitate both types of memory consolidation is consistent with previously studies in which systemic administration of G-1 enhanced spatial and contextual memory in male and female rodents (Hammond *et al*., 2009; 2012; Hawley *et al*., 2014; Lymer *et al*., 2017 Machado *et al*., 2019; de Souza *et al*., 2021). An innovative aspect of this work was the administration of G-1 and G-15 directly into the DH, allowing us to attribute the behavioral effects observed in the GPERs expressed in this brain region. Here, the 4 ng/h dose of G-1 elicited memory enhancement in OR only, whereas 8 ng/h G-1 was associated with enhanced memory in both tasks. These data suggest that OP is less sensitive to the memory-enhancing effects of G-1, in that spatial memory was not facilitated by the lower 4 ng/h dose. This result also suggests potentially important sex differences in the dose-response to G-1, given previous work from our laboratory showing that DH infusion of 4 ng/h G-1 improved memory in both OP and OR in OVX female mice (Kim *et al*., 2016). As such, it appears that OP

is less sensitive in males to G-1 at lower doses, suggesting that the behavioral effects of G-1 are partially distinct between female and male mice. It should be noted, however, that the 8 ng/h G-1 dose was not tested in our previous studies with females, so the response of females to this dose is unknown. Sex differences in phenotypical animal models for diseases associated with G-1 administration were also observed by Broughton *et al*. (2014) in a cerebral ischemia-reperfusion injury model, in which G-1 treatment was associated with worsened function and an increase in the core of infarct in male mice, whereas ovariectomized female mice experienced a reduction in neurological deficit, apoptosis, and infarct volume.

We then showed that post-training DH infusion of G-15 was associated with memory impairment both OP and OR. The doses of G-15 used (1.85 and 7.4 ng/h) were based on our lab's previous work with OVX mice in which post-training DH infusion of 7.4 ng/h, but not 1.85 ng/h, impaired memory in OP and OR (Kim *et al*., 2016). As in OVX females, we found that 7.4 ng/h impaired memory consolidation in both tasks, suggesting that activation of GPER is necessary for memory consolidation in males as it is in females. However, as with G-1, effects of the lower dose were task-dependent, in that 1.85 ng/h G-15 impaired OP but not OR. The memory-impairing effect of 1.85 ng/h in OP was surprising, given that this dose had no effects on OP in female mice (Kim *et al*., 2016), and suggest that spatial memory in males may be more dependent on GPER activation than in females. However, direct comparisons within the same study will be necessary to support conclusions about the differential sensitivity of males and females tested in OR and OP to low and high doses of G-15 and G-1. Nevertheless, the detrimental effects of DH G-15 infusion on memory consolidation observed here are consistent with those of previous systemic studies in which chronic systemic administration of G-15 impaired spatial memory in female OVX rats

submitted to a T-maze task and blocked neuroprotective effects of E2 in cell models of glutamate-induced neurotoxicity (Hammond *et al*., 2012; Gingerich *et al*., 2010).

GPER Intracellular Activation in DH is Distinct in Male Mice

Given the consistent memory-enhancing effects of 8 ng/h G-1 in both tasks, we next sought to determine which intracellular pathways might mediate effects of GPER activation in males. Previously, our laboratory demonstrated that in OVX mice, GPER activation in the DH was associated with increased levels of phospho-JNK 5 minutes after infusion and enhancement of cofilin phosphorylation after 5 and 15 minutes after G-1 infusion (Kim *et al*., 2016; 2019). Activation of both pathways in the DH was necessary for G-1 to promote OR and OP memory consolidation, and cofilin-dependent actin polymerization was necessary for G-1 to increase CA1 dendritic spine density (Kim *et al*., 2016; 2019). Our initial hypothesis was that the same mechanisms would underlie the behavioral effects observed in male GDX mice. Interestingly, even though most of the results from the behavioral tasks mirror previous findings observed in female mice, we did not find significant effects of G-1 on the levels of phospho-JNK following in the DH of GDX mice at 5, 15-, or 30-minutes post-infusion. Interestingly, levels of phospho-cofilin were significantly increased in both the vehicle and G-1 groups 30 minutes after infusion, suggesting a non-specific effect of the infusion procedure on cofilin at this time point. Although unclear what might have caused an increase in cofilin that was not observed in other antibodies at 30 minutes, one possibility is related to our vehicle solution. Here, we used 16% DMSO as our vehicle, and earlier *in vitro* studies showed that four days of constant exposure to DMSO 2% was associated with progressive reorganization of the cytoskeleton of B16 melanoma cells and an increase in the cellular content of the membrane cytoskeletal protein vinculin (Sousa-Squiavinato *et al*., 2019; Lampugnani *et al*., 1987). Alternatively, the elevated levels of phospho-cofilin in the vehicle group

could be associated with the infusion itself, as infusion-associated intracerebral bleeding or disruption of neurons and glia can lead to increased cofilin levels (Almarghalani *et al*., 2023, Van Troys *et al*., 2008). Regardless, the current data suggest that JNK activation and cofilin phosphorylation are not associated with the memory-enhancing effects of GPER. Future studies in which G-1 is co-infused with the JNK inhibitor SP600125 or actin polymerization inhibitor latrunculin-A will be necessary to better understand the involvement of these pathways and untangle the effects of G-1 in phospho-cofilin levels on GDX mice.

The unexpected JNK findings suggest key sex differences in the mechanisms regulating GPER-induced memory modulation. This is not, however, the first time that our laboratory has observed sex differences in the signaling mechanisms underlying estrogenic regulation of memory consolidation; several years ago, we found that the memory-enhancing effects of E2 depend on activation of p42 ERK in females, but not males (Koss *et al.*, 2018). Other reports have demonstrated sex differences in the role of protein kinase A (PKA) in mediating synaptic potentiation (Jain *et al.*, 2023, 2019), and that the effects of E2 on glutamatergic sensitivity depend on post-synaptic GPER in females, but not males (Oberlander and Woolley, 2016). Thus, we next explored other cell signaling mechanisms involved in estrogenic memory regulation.

The activation of GPER can trigger both genomic (transcriptional) and nongenomic (non-transcriptional) effects in neurons (Taxier *et al*., 2020). The rapid non-transcriptional changes observed with GPER activation have been attributed to kinase activity, and these effects have been demonstrated predominantly *in vitro* or female rodents (Zhang *et al*., 2020; Kim *et al*., 2019; Kim *et al*., 2016 Ortmann *et al*., 2011; Lucas *et al*., 2010). Moreover, there is still debate in the literature regarding how the rapid effects of E2 mediated by the classical estrogen receptors (ERα and ERβ) overlap with GPER regarding intracellular pathways (Arterburn & Prossnitz, 2023;

Prossnitz & Barton, 2023; Luo & Liu, 2020). A better understanding of similarities and differences between these intracellular signaling pathways might represent promising therapeutic targets and may lead to the use of drugs targeting classical or nonclassical estrogen receptors to avoid undesired side effects. Therefore, we next turned to the ERK and PI3K/Akt pathways because the phosphorylation of p42 ERK, PI3K, and Akt is necessary for E2 to enhance object recognition and spatial memory consolidation in OVX female mice (Koss *et al*., 2018; Boulware *et al*., 2013; Fan *et al*., 2010; Fernandez *et al*., 2008). However, we did not really expect G-1-induced changes in phosphorylation levels of these kinases because G-1 failed to activate them in OVX mice. Yet given previous sex differences in E2-induced kinase activation, we thought these kinases were worth examining.

As expected, we did not find any significant change in the phosphorylation levels of either ERK isoform, PI3K, or Akt. The lack of effects is consistent with our lab's previous findings in OVX females (Kim *et al*., 2016), and support the conclusions of our previous work that the ability of GPER to enhance memory consolidation does not involve activation of the ERK or PI3K/Akt pathways. As discussed above, the utilization of unique intracellular pathways for achieving similar behavioral effects might be of relevance considering future use of GPER agonist compounds in clinical practice; for example, G-1 systemic administration was not associated with proliferative effects indirectly measured by uterine weight in a previous study (Machado *et al*., 2019).

GPER Activation Enhances CREB Phosphorylation in Male Gonadectomized Mice

Despite the inability of G-1 to activate the JNK, ERK, and PI3K/Akt in the DH 5, 15, or 30 min after infusion, it did increase CREB phosphorylation in the DH of GDX males within 30 minutes. The effects of GPER activation on CREB phosphorylation are scarce, a previous *in vitro*

study from Zhang and colleagues (2020) showed that the incubation of G-1 in cumulus cells from oocytes of mice was also associated with elevation on phospho-CREB levels, and this effect faded in the presence of G-15. In female rats, the systemic injections of G-1 also increased levels of CREB phosphorylation in the hippocampus, and this effect was abolished when the rats received a PKA inhibitor ((Machado *et al.*, 2019). *In vitro* studies also demonstrated that dihydrotestosterone increase phospho-CREB via different kinases (i.e., ERK, PKA, PKC) (Nguyen *et al*., 2009). Here, we did not observe significant changes in the ratio of phospho-ERK, phospho-PI3K, or phospho-AKT following G-1 DH infusion despite the elevated levels of phospho-CREB, suggesting that these kinase pathways did not mediate the effects of G-1 on CREB. Interestingly, a previous study from our laboratory evaluating E2 effects on memory consolidation enhancement in gonadally intact and GDX male mice found that the levels of CREB phosphorylation in the DH were increased 5 minutes after E2 infusion, with no significant effect on phospho-ERK or phospho-AKT levels (Koss *et al*., 2018). Compared with these findings, the present work showed that G-1 DH infusion in male GDX mice also augmented phospho-CREB levels later, although this increase was found 30 minutes after infusion instead of 5 minutes. At the present time, it is unclear how G-1 leads to CREB phosphorylation, and the delayed effect on CREB relative to that of E2 perhaps suggests additional mechanisms aside from cell signaling. For example, E2 activates ERK-dependent histone acetylation in the DH 30 minutes after bilateral DH infusion in OVX females (Zhao *et al*., 2010), so the effects of on CREB may involve other processes as well. Additional work will be necessary in future studies to determine the mechanisms governing G-1-induced CREB phosphorylation in males.

Finally, we did not expect the levels of phospho-CREB to be significantly elevated in the Vehicle group 30 minutes following DH bilateral infusions. As described before, our vehicle group

was treated with 16% DMSO, and this concentration has been previously used by our and other laboratories (Hoeger-Bement & Sluka, 2003) in CNS administrations. In previous work, incubation of pancreatic rat cells with 2% DMSO did not increase cAMP levels, protein kinase A activity, or phosphorylated levels of CREB, CRE-modulator, and activating transcription factor-1 (ATF-1) (Kemp & Habener, 2002). Another *in vitro* study incubated Chinese hamster ovary cells with 98% DMSO for 72h and did not find a significant increase in the levels of phospho-CREB, suggesting that DMSO does not increase the phosphorylation of CREB (Hu *et al*., 2010). At this point, it is unclear why the 30-minute vehicle group displayed increased CREB phosphorylation. Importantly, however, treatment with G-1 significantly increased phospho-CREB levels beyond that of vehicle at this timepoint, supporting the conclusion that GPER agonism increases CREB phosphorylation 30 minutes after infusion.

Conclusions and Future Directions

In sum, the present study demonstrated that GPER regulates memory consolidation in GDX male mice. The GPER agonist G-1 enhanced spatial memory and object recognition consolidation in the OP and OR tasks, respectively, whereas the GPER antagonist G-15 blocked the formation of both types of memory. Effects of both compounds were dose-dependent, with OP differentially sensitive to lower doses of the drugs compared with OVX females. These findings provide new insights relative to previous studies of males that used systemic injections, in that bilateral DH infusions allowed us to pinpoint the role of GPER in memory formation to the DH. In addition, the data add to a growing literature showing that different cellular mechanisms underlie the effects of E2 and estrogen receptors on hippocampal function. Unlike in OVX females, we found that G-1 does not affect JNK or cofilin in the DH within 30 minutes, suggesting that these signaling kinases do not mediate the memory-enhancing effects of G-1 in GDX male mice. However, G-1

also did not activate the ERK or PI3K/Akt pathways, which is consistent with findings in OVX mice. Similar to E2 in OVX mice (Koss *et al.*, 2018), we found that G-1 increased CREB phosphorylation in the DH of GDX male mice 30 minutes. As such, the ability of GPER to regulate memory may be related to activation of this transcription factor.

Regarding the behavioral effects of G-1 in male GDX mice, future studies should examine other kinases that might be leading to CREB phosphorylation, for example, the Src pathway. Comparing our findings with previous work from Koss *et al.* (2018), we noticed that G-1 leads to a later increase in phospho-CREB; an interesting future study could also compare how E2 and G1 are associated with the activity of immediate early genes, such as c-fos, Egr-1, arc, and AP-1. Based on recent findings of Beamish *et al*. (2022) and other authors showing the importance of protein degradation, it would also be exciting to evaluate to which extent the modulation of the ubiquitin-proteasome signaling is affected by G-1. Moreover, the role of GPERs expressed in other brain regions involved in memory consolidation, such as the prefrontal cortex and the *nucleus reuniens*, could also be studied using diverse techniques such as DREADDs.

Together, our findings provide novel insights about the role of GPER in mediating cognitive function and suggest intriguing new sex differences in cell signaling pathways that underlie estrogenic regulation of memory. Future studies should seek to better understand the neural mechanisms through which GPER influences memory in males.

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