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## **G protein-coupled estrogen receptor (GPER) in the dorsal hippocampus regulates memory consolidation in gonadectomized male mice, likely via different signaling mechanisms than in female mice**

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### **Abstract**

Studies in ovariectomized (OVX) female rodents suggest that G protein-coupled estrogen receptor (GPER) is a key regulator of memory, yet little is known about its importance to memory in males or the cellular mechanisms underlying its mnemonic effects in either sex. In OVX mice, bilateral infusion of the GPER agonist G-1 into the dorsal hippocampus (DH) enhances object recognition and spatial memory consolidation in a manner dependent on rapid activation of c-Jun N-terminal kinase (JNK) signaling, cofilin phosphorylation, and actin polymerization in the DH. However, the effects of GPER on memory consolidation and DH cell signaling in males are unknown. Thus, the present study first assessed effects of DH infusion of G-1 or the GPER antagonist G-15 on object recognition and spatial memory consolidation in gonadectomized (GDX) male mice. As in OVX mice, immediate post-training bilateral DH infusion of G-1 enhanced, whereas G-15 impaired, memory consolidation in the object recognition and object placement tasks. However, G-1 did not increase levels of phosphorylated JNK (p46, p54) or cofilin in the DH 5, 15, or 30 minutes after infusion, nor did it affect phosphorylation of ERK (p42, p44), PI3K, or Akt. 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 a yet unknown signaling mechanism that triggers CREB-mediated gene transcription. Our findings show for the first time that GPER in the DH regulates memory consolidation in males and suggests sex differences in underlying signaling mechanisms.

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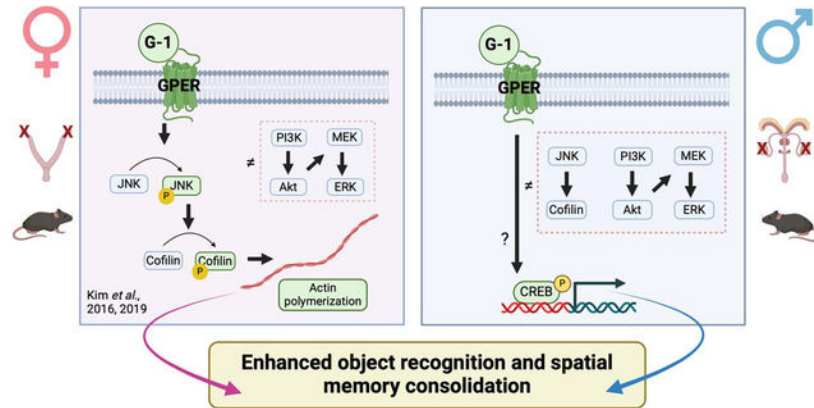
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#### Declaration of Interest

Dr. Frick is a co-founder and the Chief Scientific Officer of Estrigenix Therapeutics, Inc., a company which aims to improve women's health by developing safe, clinically proven treatments for the mental and physical effects of menopause. The rest of the authors have no conflicts of interests to declare.

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## Graphical Abstract



## Keywords

Mouse; object recognition; object placement; spatial memory; CREB; JNK; cofilin

## 1. Introduction

Although 17 $\beta$ -estradiol (E<sub>2</sub>) has long been known to promote memory and synaptic plasticity (Frick, 2015; Rocks and Kundakovic, 2023; Sheppard et al., 2018; Taxier et al., 2020; Woolley et al., 1997), the cellular and molecular mechanisms driving these effects are not fully understood in either sex. Far more is known about these mechanisms in females. For example, among ovariectomized (OVX) female mice, bilateral infusion of E<sub>2</sub> into the dorsal hippocampus (DH) before or immediately after training in object recognition, object placement, and social memory tasks enhances memory in a manner dependent on rapid phosphorylation of extracellular signal-regulated kinase (ERK) and phosphatidylinositol/Akt (PI3K/Akt) signaling in the DH, effects mediated by intracellular estrogen receptors ER $\alpha$  and ER $\beta$  binding at the plasma membrane to metabotropic glutamate receptor 1a (mGluR1a) (Boulware et al., 2013; Fernandez et al., 2008; Fortress et al., 2013; Sheppard et al., 2023). The extranuclear interactions among ER $\alpha$ , ER $\beta$ , and mGluR1a at the membrane provide a non-classical mechanism to trigger the rapid downstream cell signaling events upon which memory consolidation relies.

Another non-classical mechanism through which E<sub>2</sub> may influence memory consolidation is via the membrane estrogen receptor G protein-coupled estrogen receptor. In female rodents, GPER regulates hippocampal synaptic plasticity and memory mediated by the DH. For example, the GPER agonist G-1 increased excitatory postsynaptic potentials (EPSPs) in hippocampal slices from OVX ER $\alpha$  knockout and ER $\beta$  knockout mice (Kumar et al., 2015), and increased miniature excitatory synaptic current (mEPSC) frequency and amplitude in hippocampal slices from OVX rats (Oberlander and Woolley, 2016). Accordingly, chronic systemic administration of the GPER agonist G-1 to OVX rats enhanced hippocampal cholinergic function and improved spatial working memory in a delayed matching-to-position T-maze task, spatial reference memory in the Barnes maze, and object recognition

memory in a novelty task, whereas systemic treatment with the GPER antagonists G-15 or G-36 impaired memory in these tasks (Bai et al., 2020; Hammond et al., 2012, 2011, 2009). In addition, chronic systemic G-1 injections improved contextual fear memory in gonadally intact mature adult (12 month-old) female mice (Xu et al., 2018). G-1 also has rapid effects on memory, as acute pre-training systemic G-1 injection in OVX mice facilitated object recognition, spatial, and social recognition memories, and these effects were associated with increased density of CA1 dendritic spines 40 min after G-1 administration (Gabor et al., 2015). Acute post-training systemic G-1 injection has also been shown to reverse the negative effects of neonatal iron treatment on memory in the object placement and inhibitory avoidance tasks among gonadally intact and OVX female rats (Machado et al., 2019). As such, data from systemic treatments suggest that GPER activation promotes memory acquisition and consolidation in female rodents, although the peripheral routes of administration used make it impossible to attribute these effects to a particular brain region.

To this end, our laboratory recently established that GPER activation in the DH is necessary and sufficient for the consolidation of long-term memories by showing that bilateral DH infusion of G-1 or G-15 immediately after training in the object recognition and object placement tasks enhanced and impaired, respectively, memory consolidation in OVX mice (Kim et al., 2016). DH G-1 infusion in OVX mice also increased CA1 apical dendritic spine density within 40 minutes (Kim et al., 2019), which is consistent with effects observed after DH infusion of E<sub>2</sub> (Phan et al., 2015; Sheppard et al., 2023; Tuscher et al., 2018). Accordingly, DH infusion of E<sub>2</sub> or G-1 also increased phosphorylation of the actin binding protein cofilin within 5 minutes (Kim et al., 2019), suggesting rapid effects of E<sub>2</sub> and GPER activation on the actin cytoskeleton that support the observed increases in DH spinogenesis. Interestingly, however, the ability of G-1 to enhance memory consolidation in the object tasks or increase CA1 spine density was not dependent on DH ERK activation as with E<sub>2</sub>, but rather relied upon phosphorylation of c-Jun N-terminal kinase (JNK) (Kim et al., 2019, 2016). Moreover, E<sub>2</sub>'s effects on memory and cofilin phosphorylation were not blocked by G-15 or a JNK inhibitor (Kim et al., 2019, 2016), suggesting that cellular mechanisms through which GPER regulates memory consolidation and CA1 spine density differ from those underlying the effects of E<sub>2</sub> and agonists of ER $\alpha$  and ER $\beta$  in OVX females.

Despite the progress made thus far in understanding the role of GPER in mediating memory in females, very little is known about its influence on memory in males. GPER is expressed in the hippocampus of both female and male rodents (Brailoiu et al., 2007), although its expression in rats differs between the sexes and across the estrous cycle. For example, GPER immunoreactivity in CA1-CA3 and the dentate gyrus of rats is higher in males and estrus females than in diestrus females (Llorente et al., 2020); however, ultrastructural analyses revealed reduced GPER expression in CA1 axons and glia in estrus females relative to males and diestrus females (Waters et al., 2015). Furthermore, systemic activation of GPER in gonadally intact male rodents suggests that this receptor facilitates hippocampus-dependent memory in males. For example, acute systemic G-1 treatment in young adult male rats enhanced memory consolidation in the object recognition and inhibitory avoidance tasks when injected immediately after training, but not 3 or 6 hours after training (de Souza et al., 2021). Accordingly, immediate post-training injection of G-15 impaired object recognition memory consolidation (de Souza et al., 2021), suggesting

an important role for GPER in memory consolidation among males. Among mature adult (12-month-old) male mice, chronic systemic G-1 treatment enhanced spatial memory in the Morris water maze and contextual fear memory (Xu et al., 2018). Although these studies suggest a similar memory-promoting role for GPER in males as previously observed in females, the neural mechanisms underlying its regulatory effects are unknown, both in terms of brain regions and cell signaling pathways involved. Moreover, there is reason to believe that the cellular mechanisms mediating GPER's effects on memory may differ in males and females. For example, G-1 increased mEPSC frequency and amplitude in hippocampal slices from OVX rats, but not gonadectomized (GDX) male rats (Oberlander and Woolley, 2016), indicating sex differences in the role of post-synaptic GPER activation in regulating hippocampal glutamate sensitivity. These findings echo sex differences in the mechanisms through which E<sub>2</sub> influences glutamate neurotransmission and synaptic plasticity in rats, such that long-term potentiation (LTP) in OVX females, but not GDX males, depends on synaptic activity, cAMP-regulated protein kinase A (PKA) activation, and calcium-permeable AMPA receptors (Jain et al., 2018; Jain and Woolley, 2023). Furthermore, our laboratory previously reported that the memory-enhancing effects of E<sub>2</sub> on object recognition and spatial memory consolidation depend on p42ERK phosphorylation in OVX mice, but not gonadally intact or GDX male mice (Koss et al., 2018). Although it remains unclear which signaling mechanisms mediate E<sub>2</sub>-induced memory enhancement in males, E<sub>2</sub> did increase phosphorylation of the transcription factor CREB in both sexes (Koss et al., 2018), suggesting that E<sub>2</sub> may sex-dependently regulate the activity of kinases upstream of CREB. Collectively, these data suggest key sex differences in the cellular mechanisms that underlie E<sub>2</sub>'s effects on hippocampal synaptic plasticity and memory and raise the possibility that the mechanisms regulating GPER's effects on memory may also be sex specific.

Given the dearth of information about the role of GPER in mediating memory in males, as well as the neural mechanisms through which GPER may regulate memory, the goals of this study were to determine the extent to which dorsal hippocampal GPER regulates object recognition and spatial memory consolidation in GDX male mice and to identify cell signaling pathways, such as JNK, that might be involved. As in females, our data indicate that GPER in the DH is a key regulator of memory consolidation in males but suggest sex differences in underlying signaling mechanisms. These findings provide the first evidence that GPER in the DH modulates memory formation in males and suggest the intriguing possibility that this receptor may do so by triggering different signaling pathways in males and females.

## 2. Methods

### 2.1. Subjects

Male C57BL/6 mice (N=80) were purchased from Taconic Biosciences 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 (Teklad Rodent Diet 8604, Envigo) and water were provided *ad libitum*. Mice were handled for 30 s/day for three consecutive days before the start of behavioral testing to become accustomed to the experimenters. All procedures were conducted from 10:00

to 17:00 h in a quiet room and experimenters conducting behavioral testing were blind 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.

## 2.2. General Experimental Design Overview

Fig. 1 illustrates a schematic overview of the general experimental design used in these studies. Male mice were first bilaterally gonadectomized and bilaterally implanted with stainless steel guide cannulae aimed at each DH within the same surgical session. They were then allowed a minimum of one week to recover before the start of habituation in the object recognition (OR) and object placement (OP) tasks. Although memory consolidation in both tasks depends on intact DH function (Tuscher et al., 2018), our prior work indicates somewhat differential roles of estrogen receptors in mediating OR and OP memory in OVX mice, as DH infusion of the ER $\alpha$  antagonist MPP selectively impaired OP memory, whereas the ER $\beta$  antagonist PHTPP impaired both OR and OP memory (Kim and Frick, 2017). Thus, both tasks were included here to determine the extent to which GPER might modulate both spatial and object recognition memories. Immediately after training in the OR and OP tasks, mice were restrained gently and infused bilaterally into the DH with vehicle, the GPER agonist G-1, or the GPER antagonist G-15 (to test effects of GPER antagonism). Two experiments were conducted; one in which mice were infused with vehicle or G-1 (4 or 8 ng/hemisphere) and another in which they were infused with vehicle or G-15 (1.85 or 7.4 ng/hemisphere). For G-1, memory consolidation was evaluated 48 h later in OR and 24 h later in OP. For G-15, memory was tested 24 and 4 hours later in OR and OP, respectively. These time points were selected because vehicle-treated gonadectomized mice of both sexes spend more time with the novel or moved objects in OR and OP testing, 24 h and 4 h, but not 48 h and 24 h, respectively, after training (Kim et al., 2019, 2016; Koss et al., 2018). Thus, the longer delays allowed us to assess the possible memory-enhancing effects of G-1, whereas the shorter delays permitted testing of potential memory-impairing effects of G-15. A minimum of 14 days separated OR and OP testing, the order of which was counterbalanced within a group (each cohort included 10–12 mice/group); this interval between test bouts allowed metabolic clearance of the drugs from the brain and for any acute effects of infusion on brain structure or function to dissipate prior to the next infusion. Finally, at least ten days after the final behavioral test, mice were infused again with G-1 and the DH was collected bilaterally 5, 15, or 30 min later to determine levels of phosphorylated JNK, cofilin, ERK, PI3K, Akt, and CREB via Western blotting.

## 2.3. Surgical Procedures

At least four days after arrival in the laboratory, mice underwent bilateral orchietomy followed immediately by bilateral implantation of chronic indwelling stainless-steel guide cannulae into the DH as described previously (Kim et al., 2019, 2016; 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 orchietomy 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. Mice were then implanted with bilateral guide cannulae (22 gauge; C232G, P1 Technologies (formerly Plastics One Inc.)) aimed at each hemisphere of the DH (1.7 mm AP,  $\pm$ 1.5 mm ML, 2.3 mm DV). Dummy cannulae (C232DC, P1 Technologies) 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 observed carefully for any sign of discomfort and received  $\frac{1}{4}$  of a 2 mg Rimadyl tablet for pain relief on the first post-operative day, and as then needed.

#### 2.4. Drugs and Infusions

Post-training drug infusions were performed as described previously (Kim et al., 2016, 2019; Koss et al., 2018) by gently restraining each mouse to remove the dummy cannulae, followed by placement of an infuser into each guide cannula (C3131; DH; 28 gauge, extending 0.8 mm beyond the 1.5 mm guide). The infuser was secured to PE50 polyethylene tubing attached to a 10  $\mu$ l Hamilton syringe. Infusions were controlled by a micro infusion pump (KDS Legato 180, KD Scientific) and delivered at a rate of 0.5  $\mu$ l/min for 1 min. Our unpublished data using AlexaFlour 384 indicates that the spread of infusions using this protocol is restricted to the DH. Infusers remained in place for 1 min 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 our laboratory'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., 2019, 2016). Vehicle controls for G-1 and G-15 were 16% and 2% DMSO, respectively.

#### 2.5. 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 (Boulware et al., 2013; Fernandez et al., 2008; Tuscher et al., 2016). Both tasks were conducted in a white open field box (width, 60 cm; length, 60 cm; height, 47 cm) under dim lighting ( $\sim$ 75 lx) provided by torchiere lights spaced around the box to provide equivalent light to the corners of the box. Before the start of behavioral training, mice were handled for 30 s/day for three consecutive 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 were habituated to the empty open field box for 5 min/day for two consecutive days. During habituation, mice could freely explore the apparatus without objects present.

Following habituation, mice underwent OP or 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 object exploration in

real-time using ANYmaze tracking software (Stoelting). Exploration was counted when the mouse's nose and/or front paws were directed towards and/or touching the objects. Time spent exploring the objects and time to accumulate 30 s of object exploration were recorded. Different objects were used for OP and OR, and all objects used were counterbalanced across mice and between sides of the box to account for any potential preferences for objects or locations. 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. Immediately following training, mice were given bilateral DH infusions of vehicle, G-1, or G-15 as described above. These treatments were administered immediately post-training to pinpoint effects of G-1 and G-15 to the consolidation phase of spatial and object recognition memory formation. Mice that did not accumulate 30 s of exploration during training were re-trained 4–7 days later with different objects and were given up to three subsequent chances to successfully do so.

The interval between training and testing varied depending on the drug infused as described previously (Kim et al., 2019, 2016). For OR and OP, mice were tested using delays of 48 and 24 h, respectively, for G-1, and delays of 24 and 4 h for G-15. Longer time points were used for G-1 based on previous evidence that vehicle-treated gonadally intact male mice do not remember the location or 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; Koss and Frick, 2019), permitting observation of potential memory-impairing effects of G-15. 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).

## 2.6. Western Blotting Analysis

Tissue collection and Western blotting were performed as described previously (Kim et al., 2019, 2016; Taxier et al., 2022) to measure effects of G-1 on cell signaling proteins. Briefly, mice were infused as described above and were then cervically dislocated and decapitated 5, 15, or 30 min later for bilateral dissection of the DH on an ice-cold plate. The overlying parietal, occipital, and temporal cortices were removed using a scalpel and forceps to expose the DH, and horizontal cuts were made at a 45° angle through each side of the DH at the level of the base of the superior colliculus to isolate and remove each DH. 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 probe sonicator (Branson Sonifier 250) as described previously (Kim et al., 2019, 2016; Taxier et al., 2022). Proteins were electrophoresed on 10% Tris-HCl precast gels (Bio-Rad) and transferred to PVDF membranes (Bio-Rad). 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 Clarity Max chemiluminescent substrate (BioRad). A ChemiDoc MP gel

imager (Bio-Rad) detected signal correlating with protein expression and densitometry was performed using Image Lab Software (Bio-Rad, Image Lab version 6.0.1). Blots were then stripped with ReBlot commercial stripping buffer (Bio-Rad) 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 ( $n = 5-19/\text{group}$ ) were represented as immunoreactivity relative to 5-minute vehicle-treated controls. Treatment effects were measured within single gels.

## 2.7. Statistical Analyses

Statistical analyses were conducted using GraphPad Prism 9 (La Jolla, CA). To assess learning within each group, separate one-sample  $t$ -tests were performed within each group to determine if the time spent with the novel or moved object differed from chance (15 s) (Boulware et al., 2013; Frick and Gresack, 2003; Taxier et al., 2022; Tuscher et al., 2016). 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., 2019, 2016). The time to accumulate 30 s of object exploration was analyzed using one-way ANOVA. Normalized Western blot data were analyzed using two-way ANOVAs with Treatment (vehicle, G-1) and Time (5, 15, 30 min) as independent variables. Tukey's multiple comparisons tests were used to evaluate simple effects within columns (treatment) and rows (time) (Taxier et al., 2022). Statistical significance was determined at  $p < 0.05$ . The Shapiro-Wilk Test was used to assess the normality of our samples ( $p < 0.05$ ), and all passed this normality test. Effect sizes were calculated as Cohen's  $d$  for significant  $t$ -tests and eta squared ( $\eta^2$ ) for significant ANOVAs.

## 3. Results

### 3.1. Dorsal hippocampal GPER activation promotes memory consolidation in male mice

We first evaluated the extent to which immediate post-training DH infusion of G-1 (4 or 8 ng/hemisphere (ng/h)) could facilitate memory consolidation in GDX male mice tested 48 and 24 h after OR and OP training, respectively, to provide a direct comparison with our use of these G-1 doses and testing parameters as in OVX mice (Kim et al., 2019, 2016). Mice infused with either 4 or 8 ng G-1 spent significantly more time with the novel object than chance (4 ng/h:  $t_{(12)} = 3.64$ ,  $p = 0.003$ ,  $d = 1.00$ ; 8 ng/h:  $t_{(12)} = 6.60$ ,  $p < 0.0001$ ,  $d = 1.83$ ; Fig. 2A), suggesting that both doses enhanced OR memory consolidation in males. The main effect of Treatment was also significant in the one-way ANOVA ( $F_{(2,36)} = 12.25$ ,  $p < 0.0001$ ,  $\eta^2 = 0.405$ ), and posthoc tests revealed 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. 2A). Elapsed time to accumulate 30 s of exploration did not differ among the groups ( $F_{(2,36)} = 3.03$ ,  $p > 0.05$ ; vehicle =  $425.2 \pm 48.07$ ; 4 ng/h G-1 =  $600.8 \pm 58.55$ ; 8 ng/h G-1 =  $580.2 \pm 58$ ; Fig. 2C). In OP (Fig. 2B), only mice infused with 8 ng/h G-1 explored the moved object significantly more than chance ( $t_{(11)} = 2.3$ ,  $p = 0.04$ ,  $d = 0.67$ ). Interestingly, the 4 ng/h G-1 group spent significantly less time than chance with the moved object ( $t_{(9)} = 4.08$ ,  $p = 0.002$ ,  $d = 1.29$ ). The main effect of Treatment was significant ( $F_{(2,31)} = 5.99$ ,  $p = 0.006$ ,  $\eta^2 = 0.279$ ; Fig. 2B) due to 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 =  $300.8 \pm 23.78$ ; 4 ng G-1 =  $350.9 \pm 34.75$ ; 8 ng G-1 =  $355.4 \pm 34.39$ ; Fig. 2D). Collectively, these data suggest that GPER activation dose-dependently enhances both OR and OP memory consolidation in GDX males, such that 8 ng/h G-1 enhanced memory in both tasks, whereas the effects of 4 ng/h G-1 were task-dependent.

We next evaluated the extent to which GPER antagonism could impair 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, 2019). Accordingly, vehicle-infused GDX males spent significantly more time with the novel ( $t_{(11)} = 4.36, p = 0.0011; d = 1.26$ , Fig. 3A) and moved ( $t_{(11)} = 2.553, p = 0.0268, d = 0.73$ ; Fig. 3B) objects than chance. In contrast, mice infused with 7.4 ng/h G-15 spent chance amounts of time with the novel ( $t_{(12)} = 0.4481, p = 0.6621$ ) and moved ( $t_{(12)} = 2.16, p = 0.0517$ ) objects. 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 ( $t_{(13)} = 4.248, p = 0.001, d = 1.13$ ). These treatment effects were reflected in one-way ANOVAs, such that the main effects of Treatment were significant for both OR ( $F_{(2,36)} = 3.704, p = 0.0345, \eta^2 = 0.171$ ) and OP ( $F_{(2,34)} = 5.785, p = 0.0069, \eta^2 = 0.254$ ). Posthoc analyses indicated 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 vehicle 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 =  $457.2 \pm 46.15$ ; 1.85 ng G-15 =  $493.9 \pm 39.17$ ; 7.4 ng G-15 =  $622.2 \pm 81.76$ ; Fig. 3C) or OP ( $F_{(2,34)} = 0.04, p > 0.05$ ; vehicle =  $475.5 \pm 49.08$ ; 1.85 ng G-15 =  $464.4 \pm 75.08$ ; 7.4 ng G-15 =  $453.4 \pm 44.94$ ; Fig. 3D). Together, these data indicate that G-15 dose-dependently impaired OR and OP memory consolidation in males and suggest that spatial memory consolidation in GDX males may be more susceptible than in OVX females to the memory-impairing effects of GPER antagonism.

### 3.2. Neither JNK nor cofilin were phosphorylated by GPER activation in the male DH within 30 minutes

We next sought to identify downstream cellular mechanisms underlying GPER-induced enhancements in object recognition and spatial memory consolidation. Thus, we evaluated the impact of DH G-1 infusion on the phosphorylation of signaling kinases known to regulate the effects of GPER or  $E_2$  on memory consolidation in OVX mice. In our previous work with OVX mice, 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). As such, we first examined the extent to which bilateral DH infusion of G-1 (8 ng/h) in GDX mice affected phosphorylation of the 46kD and 54kD isoforms of JNK and the actin regulatory protein cofilin. Surprisingly, G-1 did not increase DH phosphorylation of p46 or p54 JNK at any time point (Fig. 4A,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  $\times$  Time (p46:  $F_{(2,59)} = 0.48, p = 0.62$ ; p54:  $F_{(2,59)} = 1.47, p = 0.23$ ).

For cofilin phosphorylation (Fig. 4C), the main effect of Time was significant ( $F_{(2,60)} = 13.90, p < 0.0001, \eta^2 = 0.317$ ), but not the main effect of Treatment ( $F_{(1,60)} = 0.012, p = 0.91$ ) or the Treatment  $\times$  Time interaction ( $F_{(2,60)} = 0.95, p = 0.40$ ). Tukey's multiple comparison tests to evaluate simple main effects of Time within each treatment indicated that phospho-cofilin levels were significantly higher among vehicle-treated mice 30 min after infusion relative to 5 and 15 min (30 vs 5 min: adjusted  $p = 0.0009$ ; 30 vs 15 min: adjusted  $p = 0.019$ ), and among G-1-treated mice 30 min after infusion relative to 5 min (30 vs 5 min: adjusted  $p = 0.0028$ ), suggesting likely non-specific effects 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, 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 (Kim et al., 2019, 2016). Thus, we next assessed activation of classical intracellular pathways associated with E<sub>2</sub>-induced memory consolidation in OVX mice.

### 3.3. G-1 did not activate signaling pathways in the DH of males associated with E<sub>2</sub>-induced memory enhancement in females

In OVX mice, the ability of E<sub>2</sub> to enhance memory consolidation in the OR and OP tasks depends on rapid activation of p42 ERK (but not p44 ERK) and PI3K/Akt (Fernandez et al., 2008; Fortress et al., 2013; Frick, 2015; Koss et al., 2018). However, G-1 in OVX mice does not activate these signaling pathways (Kim et al., 2016), suggesting that the memory-enhancing effects of E<sub>2</sub> and GPER involve different signaling pathways in OVX females. Nevertheless, it is possible that these pathways could be involved in the memory-enhancing effects of G-1 in GDX 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.

As in OVX mice (Kim et al., 2016), G-1 had no effects on p42 or p44 ERK phosphorylation at any timepoint in GDX males, as illustrated by null effects of Treatment (p42 ERK:  $F_{(1,61)} = 1.24, p = 0.27$ ; p44 ERK:  $F_{(1,60)} = 0.08, p = 0.78$ ), Time (p42 ERK:  $F_{(2,61)} = 0.50, p = 0.60$ ; p44 ERK:  $F_{(2,60)} = 2.00, p = 0.14$ ), and Treatment  $\times$  Time (p42 ERK:  $F_{(2,61)} = 1.89, p = 0.16$ ; p44 ERK:  $F_{(2,60)} = 2.37, p = 0.10$ ) (Fig. 5A,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  $\times$  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  $\times$  Time:  $F_{(2,47)} = 1.94, p = 0.15$ ) (Fig. 5C,D). These findings suggest that the effects of G-1 on memory in GDX males do not involve ERK or PI3K/Akt signaling.

### 3.4. G-1 increased CREB phosphorylation 30 minutes after DH infusion

CREB is a primary transcription factor required for the formation of long-term memories and for synaptic plasticity in the hippocampus and other cognitive brain regions (Barco et al., 2003; Bernabeu et al., 1997; Bevilacqua et al., 1997; Koss et al., 2018). Systemic

administration of G-1 in OVX rats increased phospho-CREB levels after 3 h (Machado et al., 2019), and DH infusion of E<sub>2</sub> increased DH phospho-CREB levels within 5 min in OVX mice and both gonadally-intact and GDX male mice (Koss et al., 2018). Thus, we next examined effects of 8 ng/h G-1 on DH CREB phosphorylation. G-1 significantly increased phospho-CREB levels in a time-dependent manner (Fig. 6A), as illustrated by main effects of Treatment ( $F_{(1,57)} = 5.08, p = 0.03, \eta^2 = 0.082$ ) and Time ( $F_{(2,57)} = 31.53, p < 0.001, \eta^2 = 0.525$ ), although the Treatment  $\times$  Time interaction ( $F_{(2,57)} = 2.77, p = 0.07$ ) was not significant. Tukey's multiple comparison tests to evaluate simple effects of Time within each treatment demonstrated that levels of phospho-CREB among G-1-infused mice were significantly elevated 30 min after infusion relative to the 5 and 15 min time points (adjusted  $p < 0.0001$  for both comparisons). Interestingly, the 30 min vehicle group also exhibited significantly elevated levels of phospho-CREB relative to the 5 min vehicle group (adjusted  $p = 0.003$ ), although the significant main effect of Treatment suggests a greater increase at this time point after G-1 infusion.

#### 4. 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 females. Considerably less work has focused on males, despite the presence of E<sub>2</sub>, estrogen receptors, and *de novo* estrogen synthesis in the male brain (Cornil et al., 2012; Frick et al., 2018; Hutson et al., 2019; Koss and Frick, 2019; Maney and Pinaud, 2011). As such, the current study was designed to provide new insights into estrogenic regulation of memory in males by specifically focusing 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., 2019, 2016). Consistent with the data from OVX females, the present findings demonstrate that pharmacological activation of dorsal hippocampal GPER by G-1 enhances object recognition and spatial memory consolidation in GDX male mice. Additionally, antagonism of GPER by G-15 impaired the consolidation of both types of memory, as observed in females (Kim et al., 2019, 2016). Unlike in previous studies of males in which G-1 and GPER antagonists were injected systemically (de Souza et al., 2021; Xu et al., 2018), G-1 and G-15 were infused here into the DH, establishing specific involvement of GPER in this brain region in males. However, the cellular mechanisms within the DH that regulate the effects of GPER in males are unclear. Unlike in females (Kim et al., 2019, 2016), DH infusion of G-1 did not enhance the phosphorylation of JNK or cofilin within 30 minutes, nor did it activate the ERK or PI3K/Akt pathways as E<sub>2</sub> does in OVX females (Fernandez et al., 2008; Fortress et al., 2013). G-1 did increase the levels of phospho-CREB in the DH 30 minutes after infusion, indicating downstream effects on gene transcription, but the cell signaling events leading to this increase remain unclear at the present time. Together, our findings indicate that GPER activation in the DH is a key regulator of object recognition and spatial memory consolidation in GDX male mice, although the intracellular signaling pathways involved appear to be distinct from those described previously in OVX female mice. As such, these

data underscore the importance of investigating sex differences in the neural mechanisms underlying hormonal regulation of memory.

#### **4.1. A role for DH GPER activation on spatial and object recognition memory consolidation in males**

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 beneficial effects of G-1 on memory consolidation in the OR and OP tasks are consistent with previous studies in which systemic administration of G-1 enhanced spatial and contextual memory in male and female rodents (Bai et al., 2020; de Souza et al., 2021; Hammond et al., 2012, 2009; Hawley et al., 2014; Lymer et al., 2017; Machado et al., 2019; Xu et al., 2018). However, the use of intrahippocampal infusions in this study allowed us to specifically pinpoint the memory-enhancing effects of G-1 to GPER in the DH. Here, the 4 ng/h dose of G-1 facilitated object recognition memory consolidation only, whereas 8 ng/h G-1 enhanced consolidation in both tasks. These data suggest that spatial memory consolidation is less sensitive to the beneficial effects of G-1 in males, in that consolidation in the OP task was not facilitated by the lower 4 ng/h dose. These findings also suggest potentially important sex differences in the dose-response to G-1, given previous work from our laboratory demonstrating that DH infusion of 4 ng/h G-1 improved memory consolidation in both the OP and OR tasks among OVX mice (Kim et al., 2016). As such, the sensitivity of spatial memory to GPER activation may differ somewhat 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 OVX mice, so the response of females to this dose is unknown. A previous study in which 1 or 5 µg G-1 was injected subcutaneously for 15 consecutive days reported that both doses improved spatial memory in the Morris water maze in mature adult intact male mice, suggesting that the dose sensitivity of spatial memory to G-1 could be specific to the OP task or related to age or the loss of gonadal hormones (Xu et al., 2018). Future work directly comparing effects of G-1 on consolidation in OP and other spatial tasks among young adult male and female mice (with and without gonads) will support more definitive conclusions about the potential reduction in OP sensitivity to GPER activation.

We next showed that immediate post-training DH infusion of G-15 impaired memory consolidation in both the OP and OR tasks. The doses of G-15 used (1.85 and 7.4 ng/h) were based on our laboratory'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 consolidation in OP and OR (Kim et al., 2016). As in OVX females, we found that 7.4 ng/h G-15 impaired 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 memory consolidation in 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, as with G-1, 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. Nevertheless,

the detrimental effects of DH 7.4 ng/h G-15 infusion on memory consolidation observed here are consistent not only with our own DH infusions in OVX mice, but also with those of previous systemic studies in which acute post-training G-15 injection impaired object recognition memory consolidation in gonadally-intact male rats (de Souza et al., 2021) and chronic minipump administration of G-15 impaired spatial memory in a T-maze among female OVX rats (Hammond et al., 2012).

Together, these data suggest that activation of GPER in the DH is necessary for the formation of recognition and spatial memories in both sexes, yet the effects of G-15 in this and other studies beg the question of what signal G-15 inhibits in OVX and castrated mice with low circulating sex steroid levels. In males, estrogens are mainly produced in the testis (Hess, 2003) and the brain, including the hippocampus (Hoyo et al., 2004; Prange-Keil et al., 2003; Hernández-Vivanco et al., 2022). Local E<sub>2</sub> synthesis is necessary for maintaining hippocampal spine synapses (Kretz et al., 2004) and hippocampal synaptic plasticity (Zhou et al., 2010). In male zebra finches, social interactions with females or exposure to other male's songs increase forebrain E<sub>2</sub> synthesis (Ramage-Healey et al., 2008), suggesting that learning triggers brain E<sub>2</sub> synthesis. Our work with OVX females suggests that object learning increases DH E<sub>2</sub> synthesis, and that infusion of the aromatase inhibitor letrozole into the DH blocks both this increase and memory consolidation in the OR and OP tasks (Tuscher et al., 2016). Thus, we hypothesize that the signal inhibited by G-15 in the DH is induced by object learning. This hypothesis is supported by our previous work in GDX male mice showing that DH letrozole infusion blocks memory consolidation in the OR and OP tasks (Hernández-Vivanco et al., 2022; Hoyo et al., 2004; Prange-Kiel et al., 2003), as well as data from hippocampal cultures showing that treatment with NMDA increases E<sub>2</sub> synthesis (Fester et al., 2016). Because our findings from OVX females suggest that E<sub>2</sub> and GPER regulate memory consolidation via divergent cell signaling pathways (Kim et al., 2016), it is unclear whether E<sub>2</sub> is the ligand whose actions are blocked by G-15, or whether other estrogens or sex steroids are the critical signal(s). This issue should be addressed in future studies.

#### **4.2. GPER activation in the DH of males did not affect phosphorylation of JNK, cofilin, ERK, PI3K, or Akt within 30 minutes**

Given the consistent memory-enhancing effects of 8 ng/h G-1 in both tasks, we next used this dose to determine which cell signaling pathways might mediate the memory-enhancing effects of GPER activation in males. In OVX mice, we previously found that G-1 increased levels of phospho-JNK in the DH 5 minutes after DH infusion and increased DH cofilin phosphorylation 5 and 15 minutes after G-1 infusion (Kim et al., 2019, 2016). Activation of both pathways in the DH was necessary for G-1 to promote memory consolidation in the OR and OP tasks, and cofilin-dependent actin polymerization was necessary for G-1 to increase CA1 dendritic spine density (Kim et al., 2019, 2016). Thus, our initial hypothesis here was that JNK and cofilin phosphorylation would be increased by G-1 in male mice. Surprisingly, even though most of the behavioral effects of G-1 mirrored our previous findings in females, G-1 in this study did not affect levels of phospho-JNK in males at any time point after DH infusion. Interestingly, DH phospho-cofilin levels 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 for other signaling proteins, one possibility is related to our vehicle solution. Here, our vehicle was 16% DMSO, and earlier *in vitro* studies showed that four days of constant exposure to 2% DMSO 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 (Lampugnani et al., 1987; Sousa-Squiavinato et al., 2019). The concentration of DMSO is an important determinant of its effects on cell membranes, as DMSO in low concentrations (<10 mol %) is associated with a significant reduction in membrane thickness, such that concentrations between 10–20 mol % are associated with increased water pore formation, and concentrations >20 mol % are associated with desorption of individual lipid molecules and disintegration of the lipid bilayer membrane structure (Gurtovenko and Anwar, 2007). It is important to note that although these effects are particularly important for the diffusion of hydrophilic molecules, they could potentially mediate the enhancement observed in phospho-cofilin levels in our vehicle group, as caveolae structural changes in the phospholipid membrane can lead to kinase activation (Mineo et al., 1998). 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 in GDX male mice. However, future studies in which G-1 is co-infused with the JNK inhibitor SP600125 or actin polymerization inhibitor latrunculin-A will be necessary to rule out involvement of these pathways more definitively.

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 E<sub>2</sub> depend on activation of p42 ERK in female, but not male, mice (Koss et al., 2018). Other reports have demonstrated sex differences in the role of PKA in mediating synaptic potentiation (Jain et al., 2018; Jain and Woolley, 2023), and that the effects of E<sub>2</sub> on glutamatergic sensitivity depend on post-synaptic GPER in female, but not male, rats (Oberlander and Woolley, 2016). Thus, we next explored other cell signaling mechanisms involved in estrogenic memory regulation.

Although work from our laboratory and others calls into question the extent to which the rapid effects of E<sub>2</sub> mediated by ER $\alpha$  and ER $\beta$  overlap with those mediated by GPER activation (Arterburn and Prossnitz, 2023; Kim et al., 2019, 2016; Luo and Liu, 2020; Prossnitz and Barton, 2023), we next turned to the ERK and PI3K/Akt pathways because the phosphorylation of p42 ERK, PI3K, and Akt is necessary for E<sub>2</sub> to enhance object recognition and spatial memory consolidation in OVX mice (Boulware et al., 2013; Fan et al., 2010; Fernandez et al., 2008; Koss et al., 2018). However, we thought it unlikely that G-1 would increase phosphorylation of these kinases in males because G-1 failed to activate them in OVX females (Kim et al., 2016). Yet given previous sex differences in E<sub>2</sub>-induced kinase activation, we thought these kinases were worth examining. As expected, G-1 did not affect phosphorylation of either ERK isoform, PI3K, or Akt. The lack of effects is consistent

with our previous findings in OVX females (Kim et al., 2016), and supports our previous conclusions that the ability of DH GPER to enhance memory consolidation does not involve activation of the ERK or PI3K/Akt pathways. The fact that GPER does not activate the same signaling pathways as E<sub>2</sub> in either sex may be advantageous in considering future use of GPER agonists to promote memory formation in clinical practice, as systemic G-1 administration is not associated with cell proliferation in tissues like the uterus (Machado et al., 2019).

#### 4.3. GPER activation enhanced CREB phosphorylation in the DH 30 minutes after infusion

Despite the inability of G-1 to activate the JNK, ERK, and PI3K/Akt pathways in the DH 5, 15, or 30 min after infusion, it did increase CREB phosphorylation in the DH of GDX males 30 minutes after infusion. Literature on the effects of GPER activation on CREB phosphorylation in the brain is scarce, but one previous study did find that CREB phosphorylation was significantly increased in the hippocampus of OVX rats 3 hours after acute systemic G-1 injection, and this effect was abolished by systemic PKA inhibitor administration (Machado et al., 2019). In cumulus cells from mouse oocytes, G-1 incubation was also associated with elevated phospho-CREB levels, and this effect was blocked by G-15 (Zhang et al., 2020). Thus, there is some precedence for increased CREB after G-1 treatment, however, the upstream signaling kinases involved are not clear. Here, G-1 did not activate ERK, PI3K/Akt, or JNK signaling in the DH, suggesting that these kinase pathways did not mediate the effects of G-1 on CREB. A previous study from our laboratory evaluating E<sub>2</sub> effects on memory consolidation in gonadally intact and GDX male mice found that the levels of CREB phosphorylation in the DH were increased 5 minutes after E<sub>2</sub> infusion, with no significant effect on phospho-ERK or phospho-Akt levels (Koss et al., 2018). Interestingly, CREB was increased by G-1 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 E<sub>2</sub> perhaps suggests additional mechanisms aside from cell signaling. For example, E<sub>2</sub> 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 E<sub>2</sub> on CREB may involve other processes as well. Immediate early genes, such as c-fos, Egr-1, arc, and AP-1, could also play a role. 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 infusion. As described before, vehicle mice received 16% DMSO, which has been previously used by our and other laboratories (Hoeger-Bement and Sluka, 2003) for intracranial administrations. In other work, incubation of pancreatic rat cells with 2% DMSO did not increase cAMP levels, PKA activity, or phosphorylated levels of CREB, CRE-modulator, and activating transcription factor-1 (ATF-1) (Kemp and Habener, 2002). Another *in vitro* study incubated Chinese hamster ovary cells with 98% DMSO for 72 h 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). Thus, it remains 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.

#### 4.4. Conclusions

In sum, the present study demonstrated for the first time that acute GPER activation in the dorsal hippocampus 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 when infused directly into the dorsal hippocampus immediately post-training. Effects of both compounds were dose-dependent, with object placement 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 dorsal hippocampal infusions allowed us to pinpoint the role of GPER in memory formation to the dorsal hippocampus. In addition, the data add to a growing literature showing that different cellular mechanisms underlie the effects of E<sub>2</sub> and estrogen receptors on hippocampal function. Unlike in OVX females, we found that G-1 does not affect JNK or cofilin in the dorsal hippocampus 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 E<sub>2</sub> in OVX mice (Koss et al., 2018), we found that G-1 increased CREB phosphorylation in the dorsal hippocampus of GDX male mice 30 minutes after infusion. As such, the ability of GPER to regulate memory may be related to activation of this transcription factor. 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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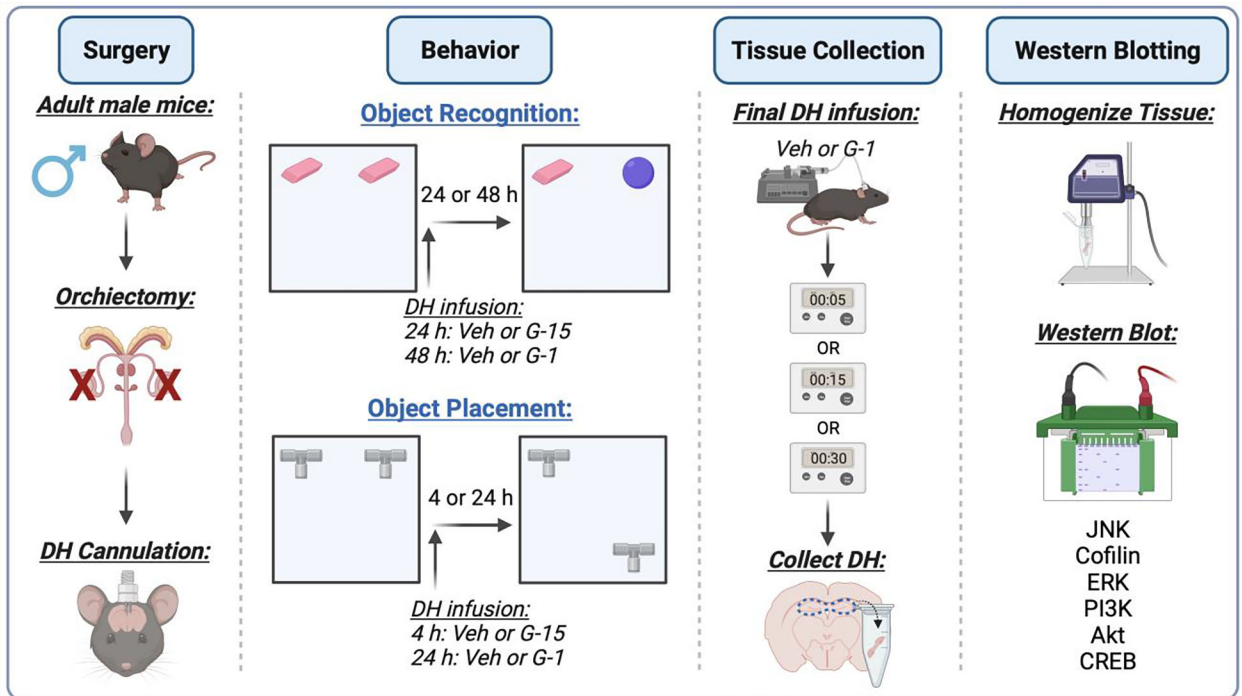
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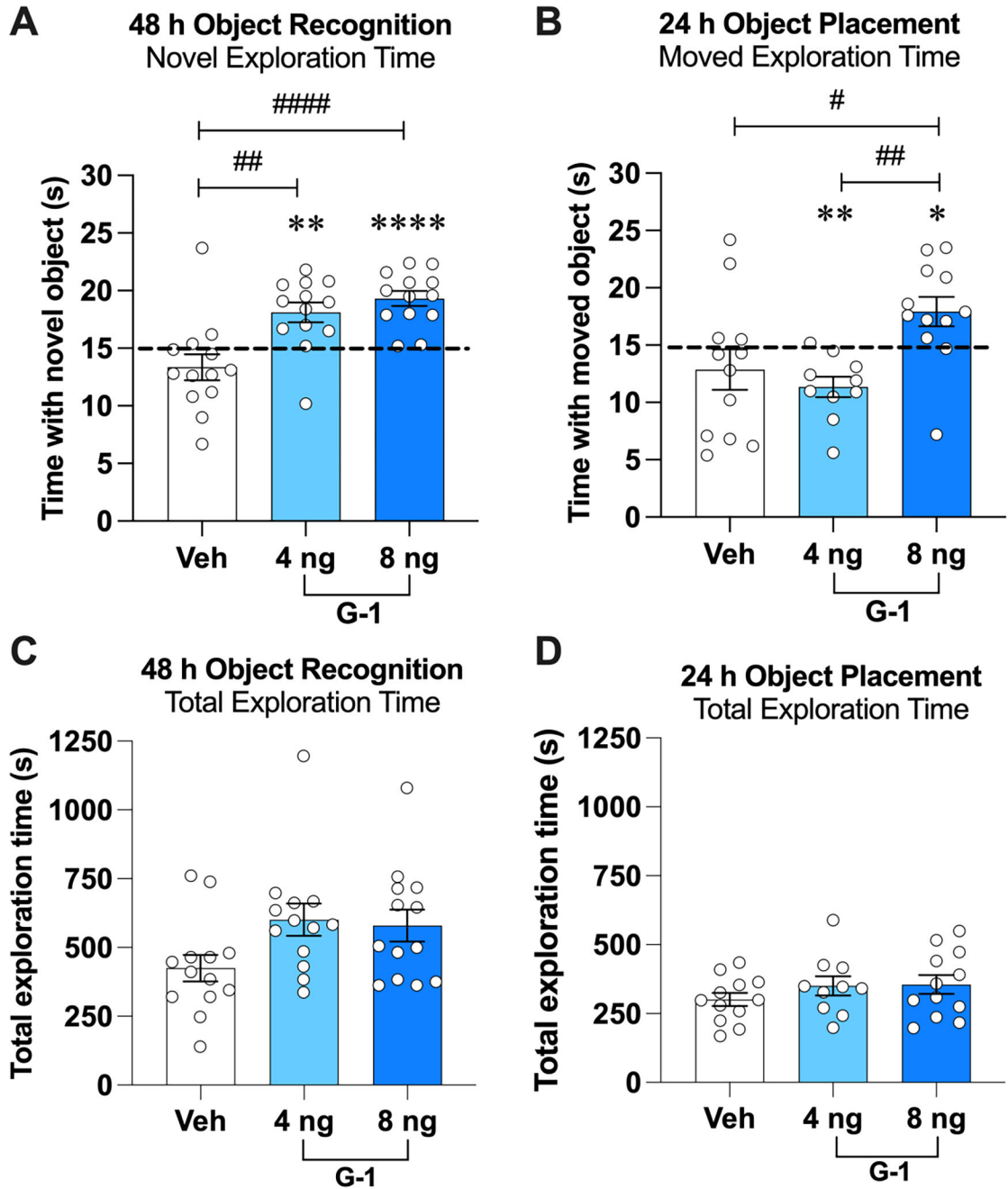
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### Highlights

- GPER agonism enhanced spatial and object recognition memory consolidation in males
- GPER antagonism impaired spatial and object recognition memory formation in males
- Dorsal hippocampal GPER promotes memory formation like in ovariectomized females
- GPER agonism did not activate dorsal hippocampal JNK, cofilin, ERK, PI3K, or Akt
- GPER agonism increased hippocampal CREB phosphorylation 30 minutes after infusion

**Fig. 1.**

Schematic diagram illustrating the general experimental design. Male C57BL/6 mice (~8 weeks old) underwent bilateral orchiectomy and implantation of dorsal hippocampus (DH) cannulae, and were then given at least 7 days to recover before the start of behavioral testing. All mice were then trained in the object recognition (OR) and object placement (OP) tasks, infused with vehicle (4% or 16% DMSO), G-1 (4 or 8 ng/hemisphere), or G-15 (1.85 or 7.4 ng/hemisphere) immediately after training (upwards arrow), and then tested at the delays indicated in the figure (see text for additional detail). Two weeks separated testing in each task and the sequence of OR and OP testing was counterbalanced within each group. Two weeks after the final behavioral test, mice were infused again and DH tissue was collected bilaterally 5, 15, or 30 min later for homogenization and Western blotting to assess levels of phosphorylated c-Jun N-terminal kinase (JNK), cofilin, extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K), Akt, and cyclic-AMP binding protein (CREB). Figure created with [BioRender.com](https://www.biorender.com).

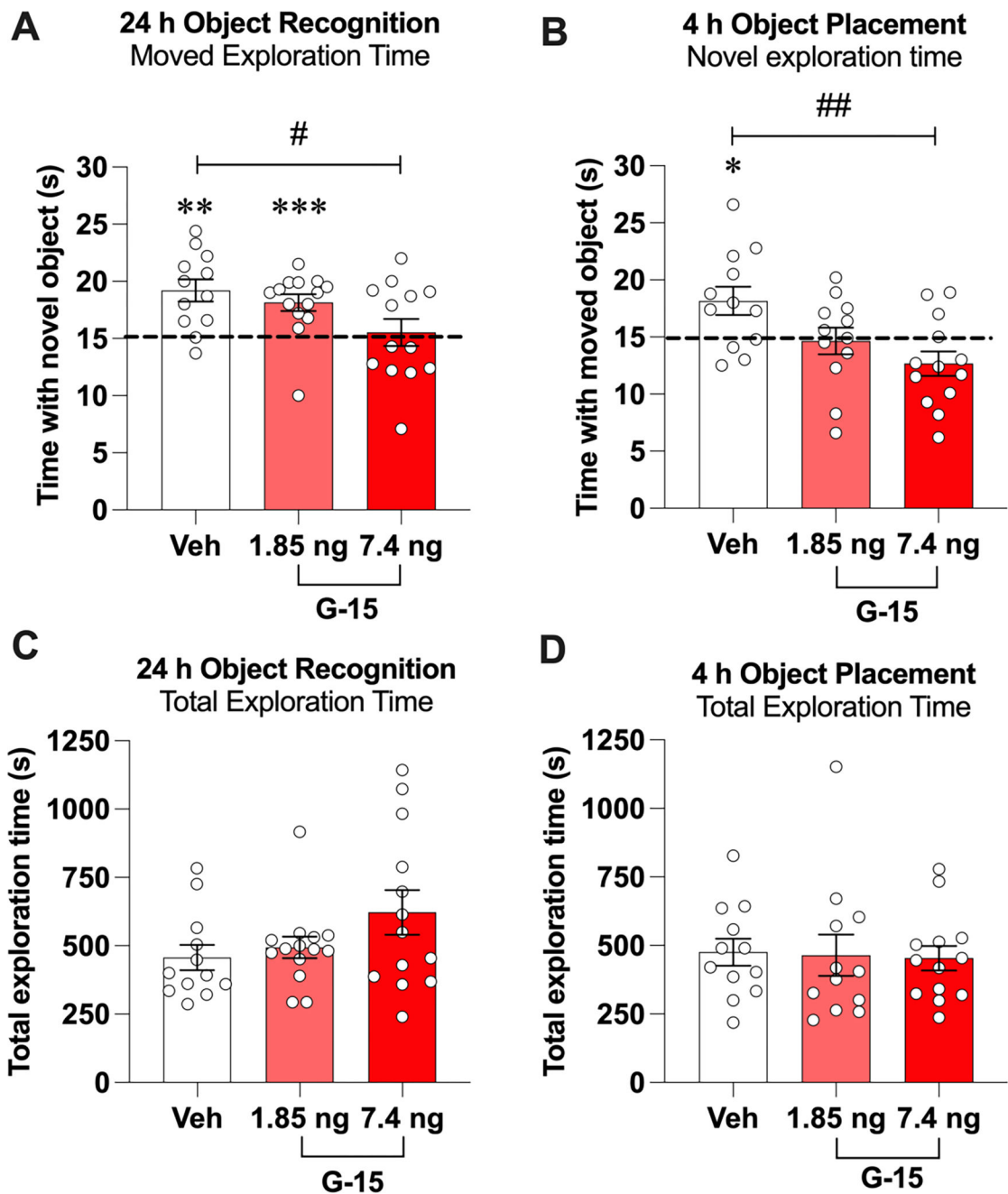


**Fig. 2.**

The GPER agonist G-1 enhanced memory consolidation in male mice. A) During OR testing, GDX 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) During OP testing, 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. The 8 ng/h G-1 group also spent more time with the moved object than the vehicle and 4 ng/h groups. C, D) The groups did not differ significantly in time to accumulate 30 s of exploration during

testing in OP or OR tasks. Circles represent individual mice and each error bar represents the mean  $\pm$  standard error of the mean (SEM) time (s) spent with the novel (OR) or moved (OP) object. (\* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\*\* $p < 0.0001$  relative to chance; # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.0001$  relative to vehicle or 4 ng/h group) (n = 12–13 mice/group).





**Fig. 3.**

The GPER antagonist G-15 impaired memory consolidation in male mice. A) In the OR task, GDX mice receiving DH infusion of 7.4 ng/h G-15 spent significantly less time than chance (dashed line) or than the vehicle group with the novel object during testing. B) During OP testing, mice receiving DH infusion of G-15 (1.85 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. C, D) There were no significant differences among groups in time to accumulate 30 s of exploration during OP or OR testing. Circles represent individual mice and each error bar represents the mean  $\pm$  SEM

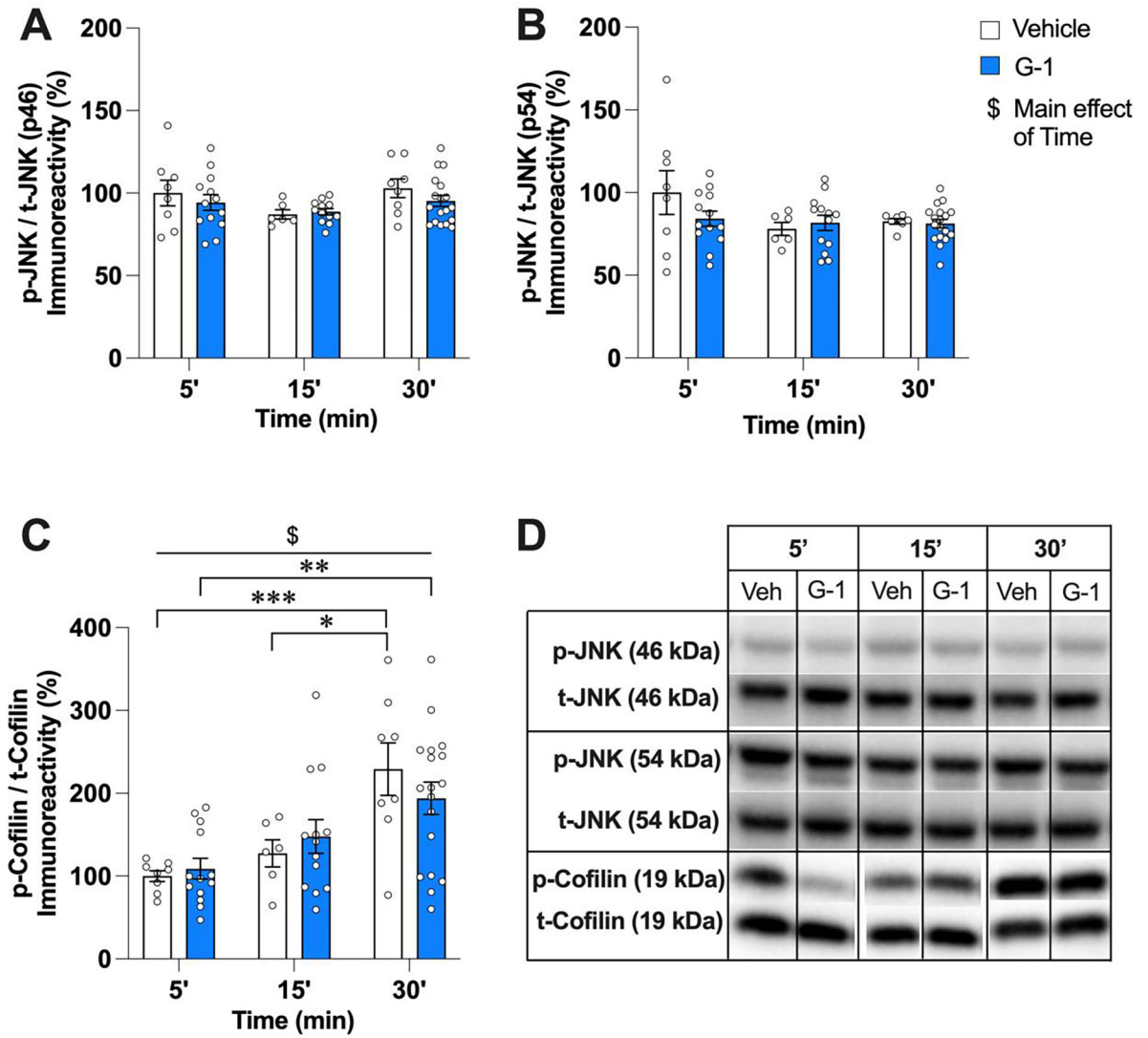
time (s) spent with the novel (OR) or moved (OP) object. (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  relative to chance; # $p < 0.05$ , ## $p < 0.01$  relative to vehicle) (n = 12–14 mice/group).

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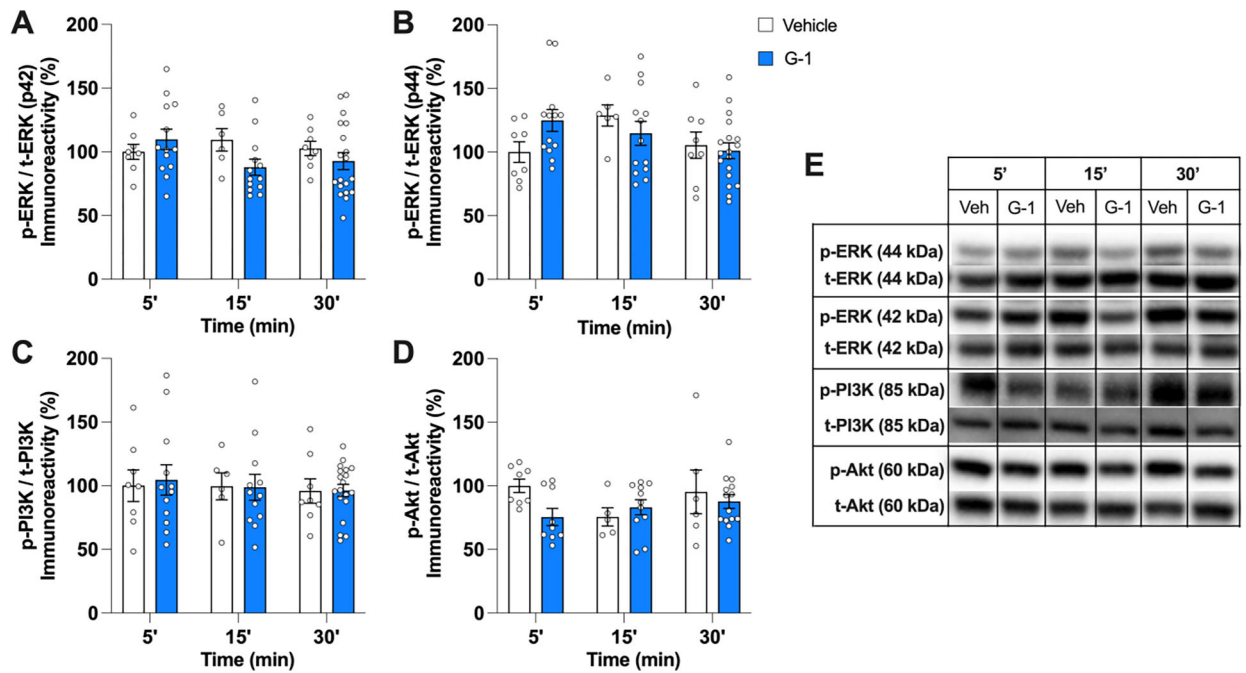
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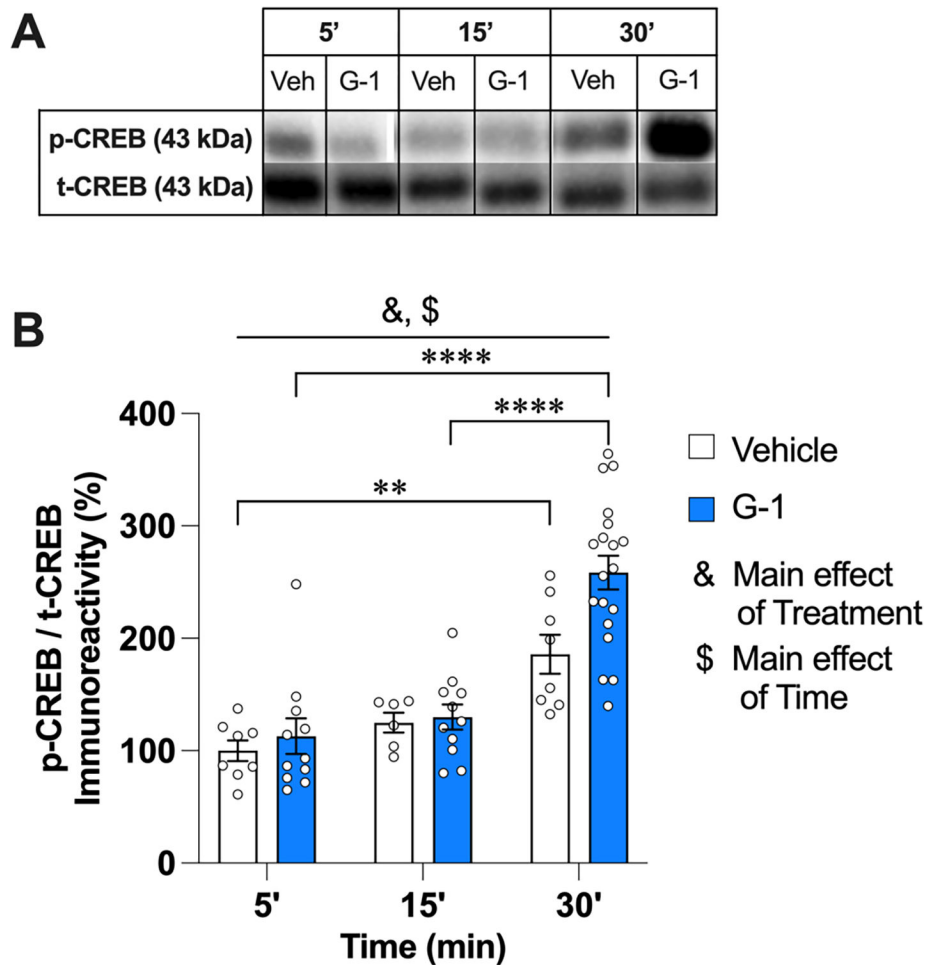
**Fig. 4.**

G-1 did not affect JNK or cofilin phosphorylation in the DH of male mice within 30 min of infusion. A, B) Infusion of 8 ng/h G-1 did not alter levels of phospho-p46 or phospho-p54 JNK at the 5, 15, or 30 min timepoints. C) Levels of phospho-cofilin were significantly higher 30 min after either vehicle or G-1 infusion, suggesting non-specific effects of infusion rather than a specific increase by GPER activation. Circles represent individual mice and each error bar represents the mean  $\pm$  SEM % change from vehicle controls (\$ represents a significant main effect of Time. Significant simple main effects of Time within treatment are represented by \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ) (n = 6–18 mice/group). D) Images of representative blots at each time point for phosphorylated and total forms of each protein.



**Fig. 5.**

G-1 did not activate ERK/PI3K/Akt pathways in the DH of male mice within 30 min of infusion. A, B) Infusion of 8 ng/h G-1 did not alter levels of p42 ERK or p44 ERK relative to vehicle 5, 15, or 30 min after infusion. C, D) G-1 also did not affect PI3K or Akt phosphorylation relative to vehicle at any time point. Circles represent individual mice; each error bar represents the mean  $\pm$  SEM % change from vehicle controls (n = 5–19 mice/group). E) Images of representative blots at each time point for phosphorylated and total forms of each protein.



**Fig. 6.** G-1 increased phospho-CREB levels in the DH 30 min after infusion. A) Images of representative blots at each time point for phosphorylated and total forms of each protein. B) Infusion of either vehicle or 8 ng/h G-1 increased CREB phosphorylation in the male DH 30 min later, although the significant Treatment effects suggests that the increase in the G-1 group was greater than that of vehicle. Circles represent individual mice; each error bar represents the mean  $\pm$  SEM % change from vehicle controls (& represents a significant main effect of Treatment; \$ represents a significant main effect of Time. Significant simple main effects of Time within treatment are represented by \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ ) (n = 6–19 mice/group).