

Dorsal Hippocampal Actin Polymerization Is Necessary for Activation of G-Protein-Coupled Estrogen Receptor (GPER) to Increase CA1 Dendritic Spine Density and Enhance Memory Consolidation

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Activation of the membrane estrogen receptor G-protein-coupled estrogen receptor (GPER) in ovariectomized mice via the GPER agonist G-1 mimics the beneficial effects of 17β -estradiol (E_2) on hippocampal CA1 spine density and memory consolidation, yet the cell-signaling mechanisms mediating these effects remain unclear. The present study examined the role of actin polymerization and c-Jun N-terminal kinase (JNK) phosphorylation in mediating effects of dorsal hippocampally infused G-1 on CA1 dendritic spine density and consolidation of object recognition and spatial memories in ovariectomized mice. We first showed that object learning increased apical CA1 spine density in the dorsal hippocampus (DH) within 40 min. We then found that DH infusion of G-1 increased both CA1 spine density and phosphorylation of the actin polymerization regulator cofilin, suggesting that activation of GPER may increase spine morphogenesis through actin polymerization. As with memory consolidation in our previous work (Kim et al., 2016), effects of G-1 on CA1 spine density and cofilin phosphorylation depended on JNK phosphorylation in the DH. Also consistent with our previous findings, E_2 -induced cofilin phosphorylation was not dependent on GPER activation. Finally, we found that infusion of the actin polymerization inhibitor, latrunculin A, into the DH prevented G-1 from increasing apical CA1 spine density and enhancing both object recognition and spatial memory consolidation. Collectively, these data demonstrate that GPER-mediated hippocampal spinogenesis and memory consolidation depend on JNK and cofilin signaling, supporting a critical role for actin polymerization in the GPER-induced regulation of hippocampal function in female mice.

Key words: actin polymerization; cofilin; GPER; hippocampus; object recognition; spatial memory

Significance Statement

Emerging evidence suggests that G-protein-coupled estrogen receptor (GPER) activation mimics effects of 17β -estradiol on hippocampal memory consolidation. Unlike canonical estrogen receptors, GPER activation is associated with reduced cancer cell proliferation; thus, understanding the molecular mechanisms through which GPER regulates hippocampal function may provide new avenues for the development of drugs that provide the cognitive benefits of estrogens without harmful side effects. Here, we demonstrate that GPER increases CA1 dendritic spine density and hippocampal memory consolidation in a manner dependent on actin polymerization and c-Jun N-terminal kinase phosphorylation. These findings provide novel insights into the role of GPER in mediating hippocampal morphology and memory consolidation, and may suggest first steps toward new therapeutics that more safely and effectively reduce memory decline in menopausal women.

Introduction

Dendritic spines are essential for synaptic plasticity and memory formation (Moser et al., 1994; Knafo et al., 2001; Nimchinsky et

al., 2002). Synapse loss is highly correlated with cognitive dysfunction in Alzheimer's disease (DeKosky and Scheff, 1990; Terry et al., 1991), a condition for which estrogen loss at menopause

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confers significantly greater risk to women. Although the potent estrogen 17 β -estradiol (E_2) has long been known to regulate dendritic spine density in the CA1 region of the hippocampus (Woolley et al., 1990; Woolley and McEwen, 1992), the molecular mechanisms mediating these effects are not well understood. E_2 -induced CA1 spine density is associated with enhanced synaptic plasticity and memory formation (Mukai et al., 2007; Inagaki et al., 2012; McClure et al., 2013; Phan et al., 2015) and changes in ovariectomized (OVXed) rats within 40 min of systemic or dorsal hippocampal treatment (Phan et al., 2011; Inagaki et al., 2012; Tuscher et al., 2016), suggesting that rapid molecular events underlie E_2 's effects on spines and memory. Indeed, rapid activation of dorsal hippocampal extracellular signal-regulated kinase (ERK) cell signaling is necessary for E_2 to increase CA1 spine density (Tuscher et al., 2016), and enhance object recognition and spatial memory consolidation (Fernandez et al., 2008; Fan et al., 2010; Fortress et al., 2013b) in OVXed mice, suggesting that E_2 may use common molecular mechanisms to mediate spinogenesis and memory consolidation.

E_2 may influence memory and spine morphology by binding to estrogen receptors, including G-protein-coupled estrogen receptor (GPER). Like E_2 , systemic dorsal hippocampal administration of the GPER agonist G-1, 1-[4-(6-bromobenzo[1,3]dioxol-5-yl)-3a,4,5,9b-tetrahydro- 3 H-cyclopenta[c]quinolin-8-yl]-ethanone (Sandia Biotech), facilitates the acquisition and consolidation of spatial and object recognition memories in OVXed rodents (Hammond and Gibbs, 2011; Gabor et al., 2015; Kim et al., 2016; Lymer et al., 2017; Paletta et al., 2018). However, the ability of hippocampally infused G-1 to enhance object recognition and spatial memory consolidation depends on phosphorylation of c-Jun N-terminal kinase (JNK), not ERK (Kim et al., 2016), suggesting that GPER and E_2 use different cell-signaling mechanisms to regulate memory formation. Yet the importance of JNK in regulating effects of GPER on CA1 dendritic spine density *in vivo* is unknown.

The actin cytoskeleton is a fundamental regulator of spine morphology (Penzes and Cahill, 2012). In hippocampal synapses, formation of the actin structure underlying the generation and enlargement of dendritic spines occurs within seconds of LTP induction, suggesting that synaptic plasticity is regulated by actin organization (Honkura et al., 2008). Interestingly, E_2 promotes hippocampal LTP *in vitro* by regulating actin polymerization (Kramár et al., 2009). The actin-binding protein cofilin is a key regulator of actin polymerization, and its inactivation via phosphorylation by signaling kinases is necessary to increase spine volume and facilitate LTP maintenance (Chen et al., 2007; Babayan and Kramár, 2013). Although cofilin inactivation is important for E_2 -induced hippocampal spine formation *in vitro* (Yuen et al., 2011; Briz and Baudry, 2014), cofilin's role in mediating effects of E_2 or GPER on CA1 spine remodeling *in vivo* is unclear. Given the close association between synapse loss and cognitive dysfunction in Alzheimer's disease, this information could inform novel treatments for arresting synapse loss and memory decline in menopausal women.

Here, we examined the involvement of JNK and actin polymerization in the effects of GPER on CA1 spine density and memory consolidation. Dorsal hippocampus (DH) GPER activation

rapidly increased CA1 spine density in a manner dependent on JNK. In contrast, E_2 's ability to increase CA1 spinogenesis did not depend on GPER activation, which is consistent with our previous behavioral findings (Kim et al., 2016). Latrunculin A, a natural toxin that inhibits actin polymerization, prevented GPER activation from facilitating CA1 spine density and memory consolidation, suggesting that GPER's effects depend on actin rearrangement. These data demonstrate a key role for actin polymerization in GPER-induced hippocampal spinogenesis and memory consolidation, and provide additional evidence that the signaling mechanisms through which GPER regulates hippocampal function are independent from those of E_2 .

Materials and Methods

Subjects. All studies used 8- to 12 week-old female C57BL/6 mice from Taconic Biosciences. After surgery, mice were housed singly in a room with a 12 h light/dark cycle, with all procedures performed between 9:00 A.M. and 6:00 P.M. Mice had *ad libitum* access to food and water. All procedures were approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee, and followed policies set forth by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Surgery. Four days after arrival, mice were bilaterally OVXed and implanted with chronic indwelling guide cannulae into the DH as described previously (Boulware et al., 2013; Fortress et al., 2014; Kim et al., 2016). Mice were secured in a stereotaxic apparatus (Kopf Instruments) and anesthetized with isoflurane gas (5% isoflurane for induction, 2% isoflurane for maintenance) in oxygen via a mask attached to the stereotax. Immediately following ovariectomy, guide cannulae (C232G; 22 gauge, Plastics One) were aimed at the DH (-1.7 mm AP, ± 1.5 mm ML, -2.3 mm DV) or both the DH and dorsal third ventricle (intracerebroventricular [ICV]; -0.9 mm AP, ± 0.0 mm ML, -2.3 mm DV). Dummy cannulae (C232DC, Plastics One) were inserted into guide cannulae to maintain patency of the cannulae. Dental cement (Darby Dental) was used to secure the guide cannulae to the skull and close the wound. Cannula placements were examined to ensure correct placement in Golgi-stained tissue (Fig. 1) or verified visually at the time of dissection for tissue taken for Western blots. Mice were given 6 d to recover before the start of behavioral testing and drug infusion.

Drugs and infusions. During infusions, mice were gently restrained while dummy cannulae were replaced with infusion cannulae (C3131; DH: 28 gauge, extending 0.8 mm beyond the 1.5 mm guide; ICV: 28 gauge, extending 1.0 mm beyond the 1.8 mm guide, Plastics One) attached to PE50 polyethylene tubing mounted on a 10 μ l Hamilton syringe. Infusions were controlled by a microinfusion pump (KDS Legato 180, KD Scientific) at a rate of 0.5 μ l/min in the DH or 1 μ l/2 min into the dorsal third ventricle using an infusion cannula as described previously (Boulware et al., 2013; Fortress et al., 2014; Kim et al., 2016). Infusion cannulae remained in place for 1 min to allow for tissue diffusion and prevent backflow through the cannula track. For triple infusion studies in which the GPER agonist G-1 was infused bilaterally into the DH in combination with infusion of a cell-signaling or actin polymerization inhibitor ICV, the inhibitor or vehicle was infused into the DH first, followed immediately by ICV infusion of G-1 or vehicle. By infusing G-1 adjacent to the DH while directly inhibiting events within the DH, this triple infusion procedure prevented tissue damage to the DH from two DH infusions in rapid succession (Fernandez et al., 2008; Zhao et al., 2010; Boulware et al., 2013; Fortress et al., 2013b; Kim et al., 2016; Tuscher et al., 2016).

G-1 and G-15 ((3aS*,4R*,9bR*)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b- 3 H-cyclopenta[c]quinoline (Sandia Biotech)) are a selective GPER agonist and antagonist, respectively, at concentrations of up to 10 μ M *in vitro* (Bologa et al., 2006; Blasko et al., 2009; Dennis et al., 2009). G-1 was dissolved in 16% DMSO in 0.9% saline and infused at a dose of 4 ng/hemisphere into the DH or 8 ng ICV (Kim et al., 2016). The vehicle control for G-1 was 16% DMSO in 0.9% saline. G-15 was dissolved in 2% DMSO and infused at a dose of 1.85 ng/hemisphere into the DH (Kim et al., 2016). The vehicle control for G-15 was 2% DMSO in 0.9% saline.

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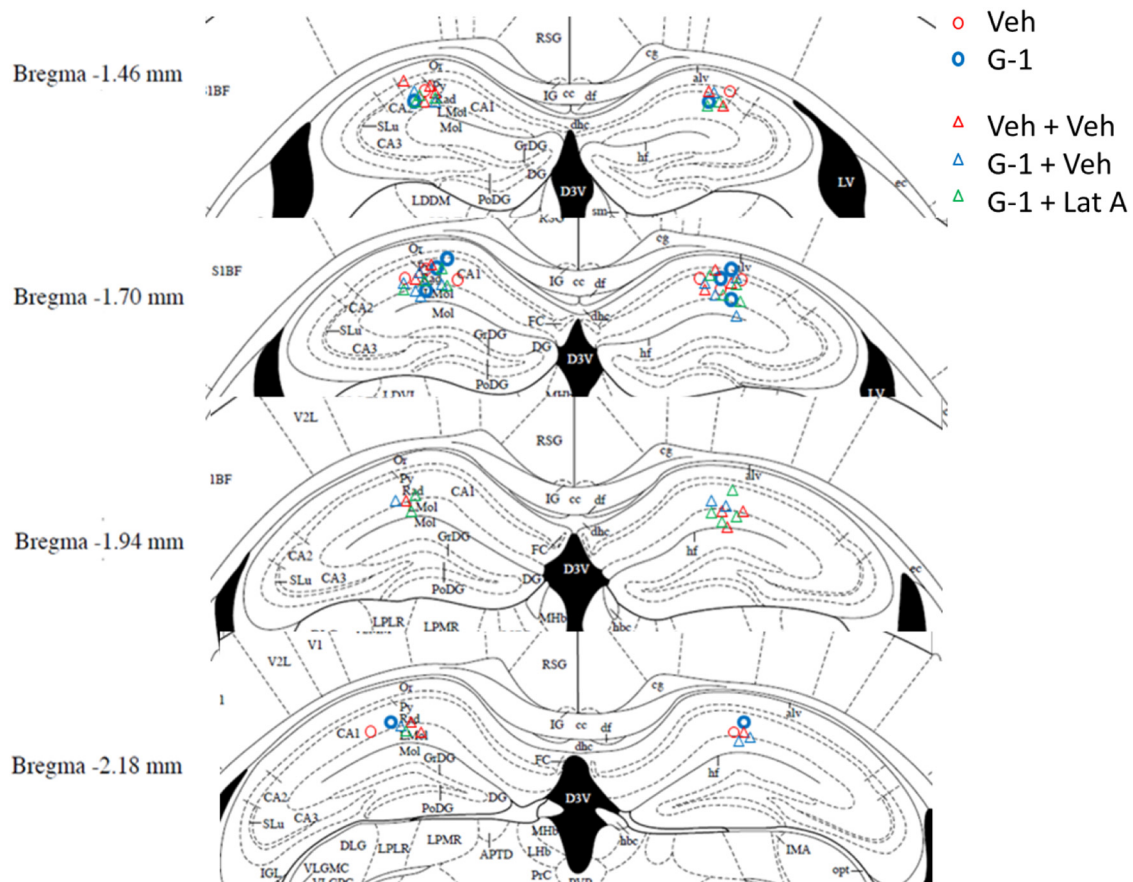


Figure 1. Schematic illustrations of dorsal hippocampal cannula placements for mice whose brains were Golgi stained. Each circle or triangle represents the approximate cannula tip placements. The position of each section relative to bregma is indicated to the left of each coronal section. Veh, Vehicle; Lat A, latrunculin A.

The JNK inhibitor SP600125 (anthra[1,9-cd]pyrazol-6(2H)-one, Sigma-Aldrich) was dissolved in 2% DMSO and infused at a dose of 2.75 ng/hemisphere into the DH (Kim et al., 2016). Cyclodextrin-encapsulated E_2 (Sigma-Aldrich) is water-soluble and is metabolized within 24 h (Pitha and Pitha, 1985; Pitha et al., 1986). E_2 was dissolved in 0.9% saline and infused at doses of 5 μ g/hemisphere into the DH or 10 μ g ICV as in previous studies (Fernandez et al., 2008; Boulware et al., 2013; Kim et al., 2016). The vehicle control for E_2 was 2-hydroxypropyl- β -cyclodextrin (Sigma-Aldrich) dissolved in 0.9% saline. Latrunculin A (Enzo Life Sciences) is a natural toxin purified from the red sea sponge *Latrunculia magnifica* that binds the ATP binding site of G-actin to prevent *de novo* actin polymerization and promote filament disassembly (Yarmola et al., 2000; Penzes and Cahill, 2012). Latrunculin A was dissolved in 1% DMSO in saline and infused into the DH at doses of 10 or 50 ng/hemisphere based on previous studies (Nelson et al., 2012; Young et al., 2014). The vehicle control was 1% DMSO dissolved in saline.

Tissue preparation and Golgi staining. Forty minutes after drug infusion, mice were cervically dislocated and decapitated, and the whole brain removed on ice. This time point was selected based on previous findings showing increased CA1 dendritic spine density 40 min after systemic G-1 treatment (Gabor et al., 2015), which was within the 30 min to 2 h time window in which DH E_2 infusion increases CA1 dendritic spine density (Tuscher et al., 2016). Rapid Golgi Stain Kit (FD NeuroTechnologies) was used for Golgi staining as described previously (Tuscher et al., 2016). Brain tissue was immersed in the impregnation solution at room temperature in the dark for 2 weeks. After the completion of impregnation, tissue was sliced into 100- μ m-thick sections using a cryostat at -30°C , and mounted on gelatin-coated microscope slides. The sections were stained per the manufacturer's instructions and coverslipped with Permount solution. Golgi-stained sections were covered by foil when not being analyzed to protect them from light exposure.

Dendritic spine counting. Dendritic spines were counted under an Olympus BX51WI microscope (100 \times with oil) using the software program NeuroLucida (MicroBrightfield, version 11.08; RRID:SCR_001775). Tertiary apical dendrites were selected from pyramidal neurons in the CA1 region of the hippocampus, based on previously established studies (Frankfurt et al., 2011; Tuscher et al., 2016). The selected dendrites were limited to those 10- to 20- μ m-long and 0.5- to 1.3- μ m-thick, were well impregnated, and whose dendrites were continuous and clearly distinguishable from adjacent cells. In brains with cannula tracks, sections selected for analysis were an average of 200 μ m (range = 0–400 μ m anterior and 0–500 μ m posterior) from a track and neurons selected for analysis were an average distance of 823 μ m (range = 517–1167 μ m) from a track. At least six neurons per brain were analyzed and 2 or 3 dendrites were analyzed per neuron. Thus, a total of at least 12 segments per brain were counted. Spine density was calculated as the number of spines/10 μ m dendrite. Dendritic spines were classified as one of four shape-based categories: branched, mushroom, thin, or stubby (Harris et al., 1992). Branched spines had multiple spine heads connected to a single spine neck. Mushroom spines had a head diameter that was at least twice as large as the neck diameter. Thin spines had a neck length that was at least twice as large as the neck diameter or larger. For thin spines, the head diameter was less than or equal to the neck diameter. Stubby spines did not have a discernible spine head. For stubby spines, the neck diameter was approximately equal to the total length of the spine.

Western blotting. Five, 15, or 30 min after infusion, mice were cervically dislocated and decapitated, and the DH dissected bilaterally on ice for Western blot analysis. Western blotting was performed as described previously (Kim et al., 2016). DH tissue was resuspended at 50 μ l/mg in lysis buffer and homogenized by sonication (Branson Sonifier 250). Homogenates were then electrophoresed on 10% Tris-HCl precast gels (Bio-Rad Laboratories) and transferred to a nitrocellulose membrane. Blots were

blocked with 5% skim milk and incubated with anti-phospho-cofilin (1:1000; Cell Signaling Technology, catalog #3313; RRID:AB_2080597) and total cofilin (1:1000; Cell Signaling Technology, catalog #3318; RRID:AB_10693596) primary antibody with the appropriate HRP-conjugated secondary antibody (1:5000; Cell Signaling Technology, catalog #7074, RRID:AB_2099233). A ChemiDoc MP gel imager (Bio-Rad Laboratories) and Image Lab software (Bio-Rad Laboratories, Image Lab version 5.2; RRID:SCR_014210) were used to perform densitometry for normalization of phospho-cofilin to total cofilin.

Object recognition (OR) and object placement (OP). OR and OP were used to test object recognition and spatial memory consolidation, respectively. Both tasks were performed as described previously (Fernandez et al., 2008; Boulware et al., 2013; Kim et al., 2016). Experimenters conducting behavioral testing were blind to treatment status, and the order of OR and OP testing was counterbalanced to minimize order effects resulting from learning, stress, or the infusion protocol. Handling, habituation, and training for both tasks were identical. Before the beginning of behavioral training, mice were handled (30 s/d) for 3 d to habituate them to the experimenters. On the second day of handling, mice were familiarized to objects by placing a small Lego in their home cage; this Lego was removed just before the start of training. Mice were then habituated in an empty white arena (width, 60 cm; length, 60 cm; height, 47 cm) for 5 min/d for 2 d. On the training day for each task, two identical objects were placed near the upper right and left corners of the arena. Mice remained in the arena until they had accumulated a total of 30 s exploring the objects. Immediately after training, mice were removed from the arena and infused. During testing, one familiar object was replaced by a novel object (OR) or was moved to a new location in the testing arena (OP). For OR, a 24 h delay was used to test the memory-impairing effects of latrunculin A and a 48 h delay was used to test the memory-enhancing effects of G-1 because young vehicle-infused OVXed mice remember the training objects after 24 h, but not 48 h (Fernandez et al., 2008; Boulware et al., 2013; Kim et al., 2016). For OP, we used a 4 h delay to test the memory impairing effects of latrunculin A and the 24 h delay to test memory enhancing effects of G-1, based on previous studies showing that vehicle-infused OVXed mice remember object locations after 4 h, but not 24 h (Fernandez et al., 2008; Boulware et al., 2013; Kim et al., 2016). Two weeks elapsed between behavioral tests to allow for any acute effects of drug infusion to dissipate before the next infusion. Different objects were used for OP and OR. For both tasks, time spent with the objects and elapsed time to accumulate 30 s of exploration were recorded using ANY-maze tracking software (ANY-maze, RRID:SCR_014289).

Experimental design and statistical analysis

Experiment 1. Experiment 1 examined whether object training itself regulates spine density in the CA1 region of the hippocampus among OVXed mice. One week after surgery, mice underwent habituation and object training with two identical objects, followed 40 min later by brain tissue collection for Golgi staining. Mice trained with objects ($n = 9$) were compared with home cage controls ($n = 8$) and yoked controls ($n = 11$). Yoked control mice performed the same habituation described above for object trained mice but were not presented with objects during the training phase. These mice remained in the empty arena for the average duration as trained mice (8 min). Home cage controls remained in their home cages for the duration of the study. Object-trained and yoked control mice were killed 40 min after removal from the arena to examine learning-induced changes in CA1 apical spine density.

Experiments 2a and 2b. Experiment 2a tested effects of DH G-1 infusion on dendritic spine density and cofilin phosphorylation. This experiment required three sets of OVXed mice. The first received vehicle ($n = 5$) or G-1 ($n = 5$) infusion, and their brain tissue was collected 40 min later for spine analysis as described above. The second set of mice received object training and then vehicle ($n = 12$) or G-1 ($n = 11$) was infused into DH right after the training. Forty minutes later, the brains were collected for spine analysis. The third set of mice was killed 5 ($n = 5$), 15 ($n = 5$), or 30 min ($n = 5$) after G-1 infusion and were compared with vehicle-infused mice ($n = 5$) killed at each time point. The DH was dissected immediately for Western blot analysis of cofilin phosphorylation. Experiment 2b examined whether GPER activation or JNK cell

signaling is important for the G-1-induced alterations in cofilin phosphorylation and CA1 spine density observed in Experiment 1a. The GPER antagonist G-15 was used to ensure that G-1 effects depended on GPER activation, and the JNK inhibitor SP600125 was used to examine whether these effects also depended on JNK signaling. A new set of mice was OVXed, implanted with ICV and DH guide cannulae, and after recovery, received ICV and DH infusions, respectively, of vehicle + vehicle ($n = 11$), G-1 + vehicle ($n = 11$), G-1 + G-15 ($n = 10$), or G-1 + SP600125 ($n = 11$). Brain tissue was collected and processed for spine analysis 40 min later. Other mice were infused with vehicle + vehicle ($n = 6$), G-1 + vehicle ($n = 5$), G-1 + G-15 ($n = 6$) or vehicle + vehicle ($n = 8$), G-1 + vehicle ($n = 7$), G-1 + SP600125 ($n = 7$), and DH tissue was collected 5 min later for Western blot analysis of cofilin phosphorylation.

Experiment 3. This experiment tested the effects of E_2 on cofilin phosphorylation and determined whether E_2 -induced cofilin phosphorylation depends on GPER activation. To establish effects of E_2 on cofilin phosphorylation, OVXed mice were killed 5 ($n = 6$), 15 ($n = 6$), or 30 min ($n = 6$) after E_2 infusion and compared with vehicle-infused mice killed at each time point ($n = 6$). DH tissue was collected for Western blot analysis of cofilin phosphorylation. To determine whether GPER activation is required for E_2 to regulate cofilin phosphorylation, another set of OVXed mice was infused with vehicle + vehicle ($n = 7$), E_2 + vehicle ($n = 7$), or E_2 + G-15 ($n = 6$), and the DH was dissected 5 min later for Western blot analysis of cofilin phosphorylation.

Experiments 4a and 4b. Experiment 4a examined the extent to which inhibition of actin polymerization impairs hippocampal memory consolidation. As described above, mice were OVXed and implanted with bilateral DH cannulae, trained in OR and OP, and then bilaterally infused immediately after training with vehicle (OP, $n = 7$; OR, $n = 9$) or one of two doses of latrunculin A: 10 ng/hemisphere (OP, $n = 7$; OR, $n = 10$) or 50 ng/hemisphere (OP, $n = 8$; OR, $n = 11$). Experiment 4b examined whether actin polymerization is crucial for GPER-mediated CA1 spine density alterations and memory enhancement. A behaviorally subeffective dose of latrunculin A that had no effect on OR or OP on its own was used in this experiment to ensure that any effects in combination with G-1 were due to an interaction between the two compounds (Fernandez et al., 2008; Boulware et al., 2013; Fortress et al., 2013b; Kim et al., 2016). As described above, mice were OVXed and implanted with DH and ICV cannulae, and then trained in OR and OP after recovery. Immediately after training, mice received ICV and DH infusions, respectively, of vehicle + vehicle (OP, $n = 11$; OR, $n = 11$), G-1 + vehicle (OP, $n = 12$; OR, $n = 10$), or G-1 + latrunculin A (OP, $n = 11$; OR, $n = 9$). OR and OP retention was tested 48 and 24 h later, respectively. Two weeks later, mice were infused with vehicle + vehicle ($n = 9$), G-1 + vehicle ($n = 10$), or G-1 + latrunculin A ($n = 10$), and then cervically dislocated and decapitated 40 min later. Whole brains were collected, and Golgi impregnated as described above.

Statistical analysis. Statistical analyses were conducted using GraphPad Prism 6 software (RRID:SCR_002798). Dendritic spine and Western blot data in all studies were analyzed with one-way ANOVA followed by Fisher's LSD *post hoc* tests or *a priori t* tests to examine treatment effects and between-group differences. For the OR and OP tasks, treatment differences among groups were assessed using one-way ANOVAs followed by Fisher's LSD *post hoc* tests. One-sample *t* tests were also used to determine whether the time spent with each object significantly differed from chance (15 s), which demonstrates evidence of learning within a treatment group. Statistical significance was determined as $p \leq 0.05$ for all tests, except for *a priori t* tests, whose significance was set at a Bonferroni-corrected $p < 0.0167$.

Results

Dendritic spine density in the hippocampus is altered by object training

Previous results from our laboratory showed that DH infusion of E_2 rapidly increases CA1 dendritic spine density via the same cell-signaling pathways critical for E_2 to enhance object memory consolidation (Tuscher et al., 2016), suggesting that object train-

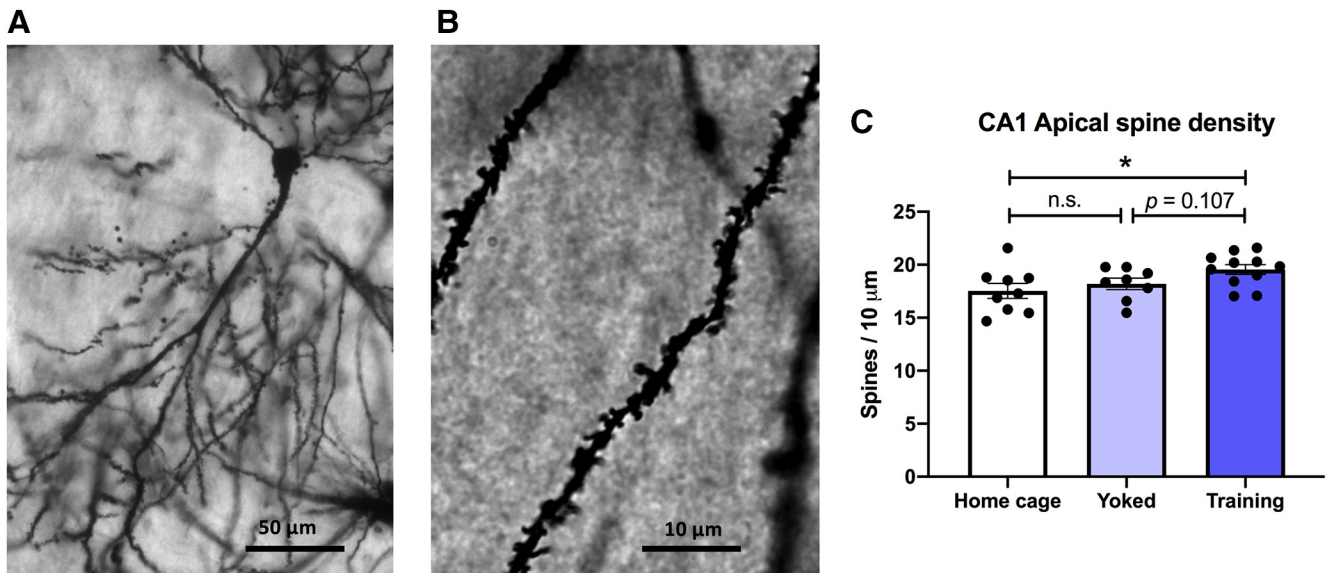


Figure 2. Learning-induced changes in CA1 dendritic spine density. *A, B*, Photomicrograph of Golgi-impregnated CA1 pyramidal cells at $20\times$ (*A*) and $100\times$ (*B*) under oil. *C*, Forty minutes after training, CA1 apical spine density was significantly increased in the object training group only, suggesting that the increased CA1 apical spine density is induced by object training. Error bars indicate mean \pm SEM. * $p < 0.05$, relative to home cage control. n.s. = not significant.

ing itself might regulate dendritic spine density. Therefore, we first sought to determine whether object training influences CA1 dendritic spine density in OVXed mice. Representative Golgi-stained CA1 pyramidal neurons are illustrated in Figure 2*A, B*. As illustrated in Figure 2*C*, the density of apical spines on CA1 tertiary dendrites in object trained mice was significantly higher than that of home cage controls; this finding was supported by a significant main effect of training ($F_{(2,25)} = 3.507$, $p = 0.0454$) and group difference ($p = 0.016$) between the object training and home cage groups. Spine density in the yoked control group was not statistically different from either the home cage ($p = 0.439$) or training ($p = 0.107$) groups.

GPER activation regulates CA1 dendritic spine density and hippocampal cofilin phosphorylation in a JNK-dependent manner

We next examined the effects of G-1 infusion on CA1 dendritic spine density. Analysis of Golgi-stained tissue revealed that DH infusion of G-1 significantly increased total CA1 apical spine density relative to vehicle 40 min after infusion ($t_{(8)} = 3.056$, $p = 0.0157$; Fig. 3*A*). To determine the effects of G-1 on specific spine types, we recounted the same dendritic segments and categorized spines as thin, stubby, mushroom, or branched as described previously (Harris et al., 1992). A two-way ANOVA (spine type \times treatment) revealed significant main effects of treatment ($F_{(1,30)} = 9.348$, $p = 0.0047$; Fig. 3*B*) and spine type ($F_{(4,30)} = 415.9$, $p < 0.0001$), and a significant interaction ($F_{(4,30)} = 4.298$, $p = 0.0072$). As before, total spine number was increased by G-1 (Fig. 3*B*; $p = 0.0018$). However, this effect was driven by a selective effect of G-1 on mushroom spines, as this was the only spine subtype in which density was significantly increased relative to vehicle (Fig. 3*B*; $p = 0.0013$). To determine whether G-1 might also potentiate spinogenesis in behaviorally trained mice, we then examined effects of DH G-1 infusion on spine density 40 min after completion of object training and G-1 infusion. The main effect of treatment was significant ($t_{(21)} = 2.402$, $p = 0.0256$; Fig. 3*C*), suggesting that post-training G-1 infusion significantly increased total CA1 apical spine density. Again, we then recounted

the same dendritic segments to assess effects on spine subtypes. Although raw values were a bit lower than those in Figure 3*A, B*, likely due to slight differences in batch and experimenter, the pattern of data was the same. A two-way ANOVA (spine type \times treatment; Fig. 3*D*) revealed significant main effects of treatment ($F_{(1,103)} = 9.958$, $p = 0.0021$) and spine type ($F_{(4,103)} = 1278$, $p < 0.0001$), without a significant interaction ($F_{(4,103)} = 1.998$, $p = 0.1003$). As was the case with behaviorally naive mice (Fig. 3*B*), only total spine number (Fig. 3*D*; $p = 0.0007$) and mushroom spine number (Fig. 3*D*; $p = 0.0248$) were significantly increased by G-1. Because mushroom spines are considered mature (Tada and Sheng, 2006; Rochefort and Konnerth, 2012), these data suggest that G-1 selectively increases the density of mature spines on apical CA1 pyramidal dendrites.

Next, we examined the effects of G-1 on cofilin phosphorylation. DH tissue was dissected for 5, 15, or 30 min after DH infusion of vehicle or G-1. The main effect of treatment was not significant ($F_{(3,16)} = 2.552$, $p = 0.0921$; Fig. 4*A*), but *a priori* t tests relative to vehicle indicated a significant increase in cofilin phosphorylation following G-1 treatment relative to vehicle 5 and 15 min after infusion (5 min, $t_{(8)} = 3.818$, $p = 0.0051$; 15 min, $t_{(8)} = 2.311$, $p = 0.0496$; Figure 4*A*). After Bonferroni correction for multiple comparisons, the difference at 5 min remained significant. Phospho-cofilin levels returned to baseline 30 min after G-1 infusion. These data suggested that GPER may affect hippocampal dendritic spine density via cofilin signaling.

To confirm that the G-1-induced phosphorylation of cofilin occurs via GPER activation, we coinfused G-1 with G-15, a selective GPER antagonist. To ensure that any effects of combined treatment were not simply due to a G-15-induced memory impairment, we used a dose of G-15 that we previously showed does not affect memory consolidation (Kim et al., 2016). DH tissue was collected 5 min after infusion due to robust effects at this time point (Fig. 4*A*). The significant main effect of treatment ($F_{(2,14)} = 13.83$, $p = 0.005$; Fig. 4*B*) was driven by the G-1-induced increase in cofilin phosphorylation; phospho-cofilin levels were significantly higher than vehicle in the group receiving G-1 ($p = 0.0002$), but not G-1 + G-15 ($p = 0.1639$). The G-1 group also

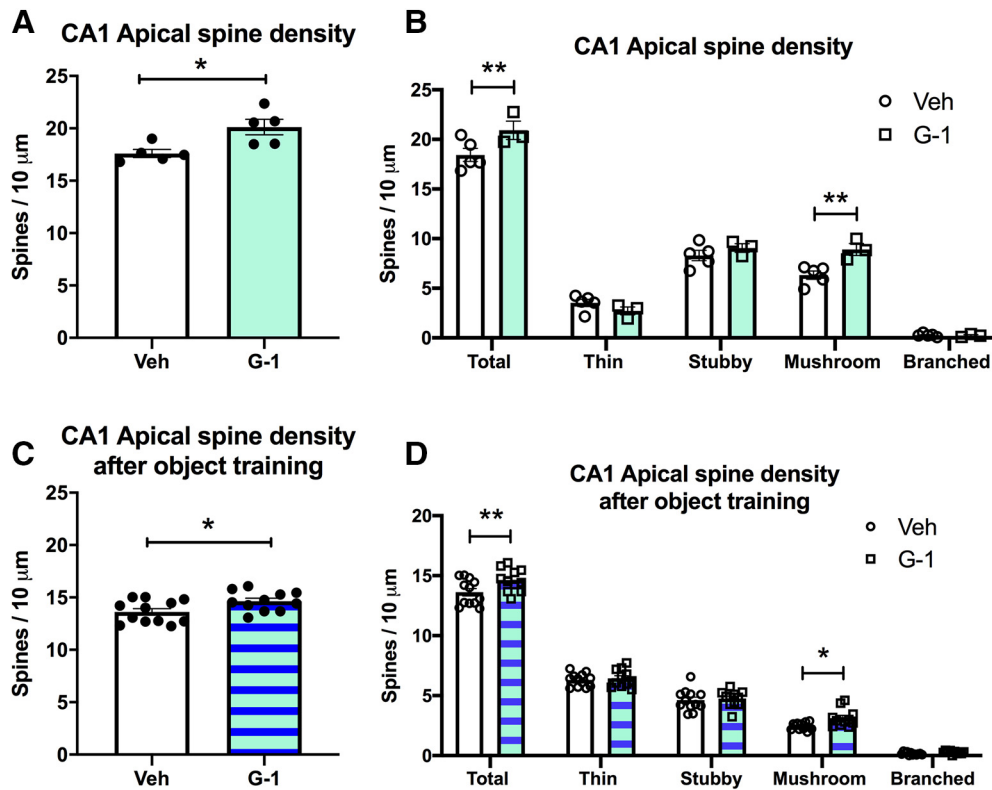


Figure 3. G-1 infusion induced spine density alterations. **A**, DH infusion of 4 ng/hemisphere G-1 into the DH increased apical CA1 spine density relative to vehicle 40 min later. **B**, Reanalysis of the dendritic segments from **A** revealed that G-1 not only increased total apical spine density, but also significantly increased mushroom-type spines relative to vehicle (Veh). **C**, Consistent with **A**, G-1 infusion (4 ng/hemisphere) administered immediately after object training significantly increased apical CA1 spine density relative to vehicle 40 min later. **D**, Similar to behaviorally naive mice, G-1 infusion in object-trained mice significantly increased mushroom-type spines relative to vehicle. Error bars indicate mean \pm SEM. * $p < 0.05$; ** $p < 0.01$; relative to vehicle control.

exhibited significantly higher levels than the G-1 + G-15 group ($p = 0.0022$), indicating that G-15 blocked the effects of G-1. To determine the importance of JNK signaling in GPER-mediated cofilin phosphorylation, we coinjected G-1 with a behaviorally subeffective dose of the selective JNK inhibitor SP600125 (Kim et al., 2016). Again, G-1 infusion increased DH cofilin phosphorylation, and DH infusion of SP600125 blocked these effects ($F_{(2,19)} = 5.031$, $p = 0.0148$; Fig. 4C). The G-1 group exhibited significantly higher phospho-cofilin levels than the vehicle ($p = 0.0057$) and G-1 + SP600125 ($p = 0.0261$) groups, who did not differ from each other ($p = 0.539$).

We next investigated the importance of GPER activation and JNK signaling in G-1-mediated dendritic spine density alterations. The main effect of treatment was significant ($F_{(3,39)} = 6.680$, $p = 0.001$; Fig. 4D), again due to the spinogenic effects of G-1. The vehicle, G-1 + G-15, and G-1 + SP600125 groups did not differ significantly from each other. In contrast, G-1 significantly increased CA1 apical spine density relative to the vehicle ($p = 0.0001$), G-1 + G-15 ($p = 0.008$), and G-1 + SP600125 ($p = 0.007$) groups (Fig. 4D), suggesting that G-1-induced spine density changes are dependent on GPER activation and JNK signaling. These findings are consistent with our previous work showing an essential role for GPER activation and JNK signaling in G-1-mediated hippocampal memory consolidation (Kim et al., 2016). Collectively, the data from this series of experiments suggest that GPER activation regulates CA1 dendritic spine density and memory consolidation via JNK- and cofilin-regulated alterations in actin polymerization.

GPER activation is not required for E_2 -induced cofilin phosphorylation

Little is known about mechanisms underlying E_2 - or estrogen receptor-induced changes in actin polymerization *in vivo*. Although it has been suggested that $ER\alpha$ and $ER\beta$ play a role in cofilin-related actin polymerization signaling (Briz and Baudry, 2014), a potential role for GPER activation in E_2 -mediated cofilin-actin polymerization signaling has not yet been examined. Because we have previously demonstrated that E_2 -mediated hippocampal memory consolidation is independent of GPER (Kim et al., 2016), we examined both the effects of DH E_2 infusion on cofilin phosphorylation and whether GPER activation is required for E_2 -mediated cofilin signaling alterations in the DH. OVXed mice received bilateral DH infusions of vehicle or E_2 (5 μ g/hemisphere), and then DH tissue was dissected for Western blotting 5, 15, or 30 min later. The main effect of treatment was not significant ($F_{(3,20)} = 2.149$, $p = 0.1259$; Fig. 5A), but an *a priori* Bonferroni-corrected *t* test revealed that E_2 infusion increased cofilin phosphorylation relative to vehicle 5 min after infusion ($t_{(10)} = 3.148$, $p = 0.0104$). Phospho-cofilin levels were not significantly different from vehicle 15 ($p = 0.0764$) and 30 ($p = 0.3846$) min after E_2 infusion. These data suggest that E_2 rapidly and transiently increases cofilin signaling in the DH.

To examine a role for GPER activation in E_2 -induced phosphorylation of cofilin, we coinjected E_2 with G-15. The main effect of treatment was significant ($F_{(2,17)} = 4.499$, $p = 0.027$; Fig. 5B), with E_2 increasing cofilin phosphorylation relative to vehicle ($p = 0.05$). However, G-15 did not block this effect, as the E_2 + G-15 group had significantly higher levels than the vehicle ($p =$

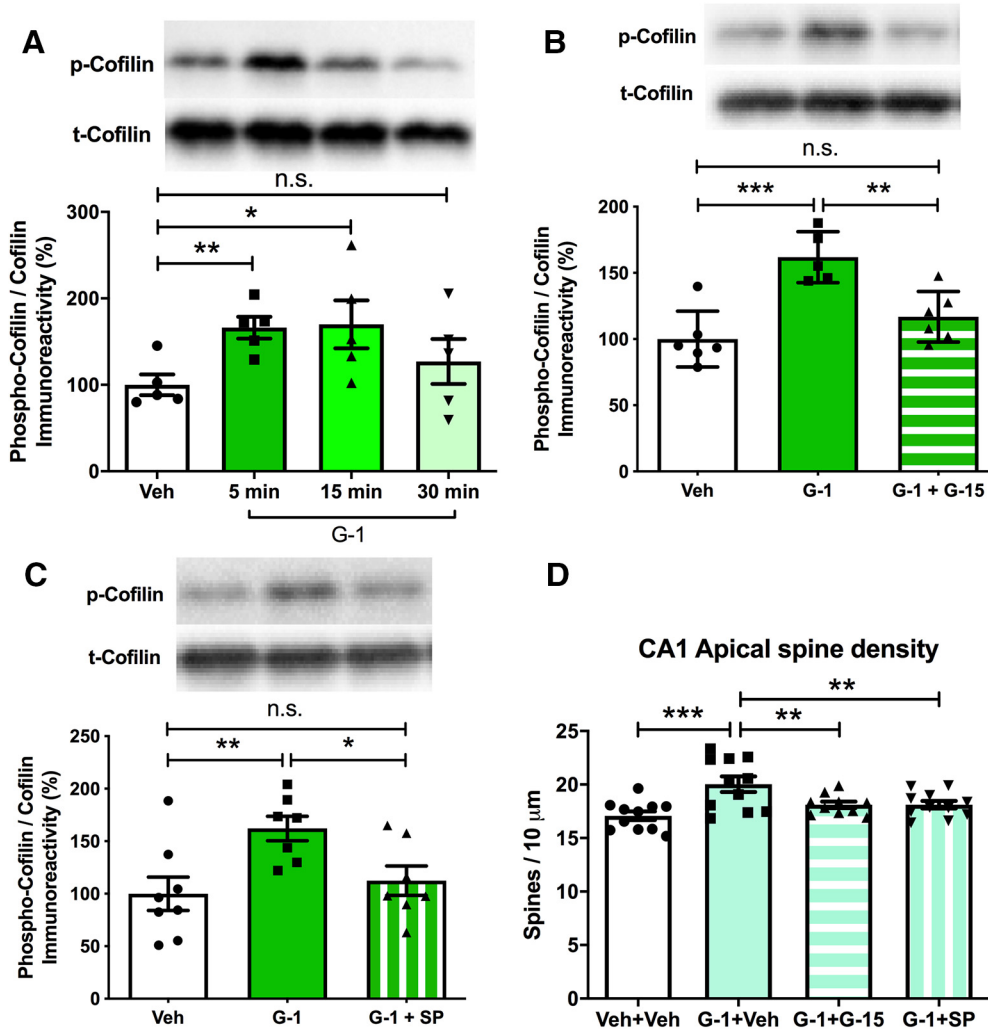


Figure 4. G-1-induced cofilin phosphorylation and spine density changes are dependent on GPER activation and JNK signaling. **A**, G-1 (4 ng/hemisphere) infusion increased DH cofilin phosphorylation relative to vehicle (Veh) 5 and 15 min after DH infusion. **B**, ICV infusion of 8 ng G-1 significantly increased levels of phospho-cofilin 5 min later. These effects were blocked by DH infusion of the GPER antagonist G-15 (1.85 ng/hemisphere). **C**, The G-1-induced increase in phospho-cofilin was also blocked by DH infusion of SP600125 (SP; 2.75 ng/hemisphere). **D**, ICV infusion of 8 ng G-1 significantly increased apical CA1 spine density relative to vehicle 40 min after infusion. DH infusion of either G-15 (1.85 ng/hemisphere) or SP (2.75 ng/hemisphere) blocked this increase. Insets, Representative Western blots. Error bars indicate mean \pm SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; relative to vehicle control or G-1-treated group. n.s. = not significant.

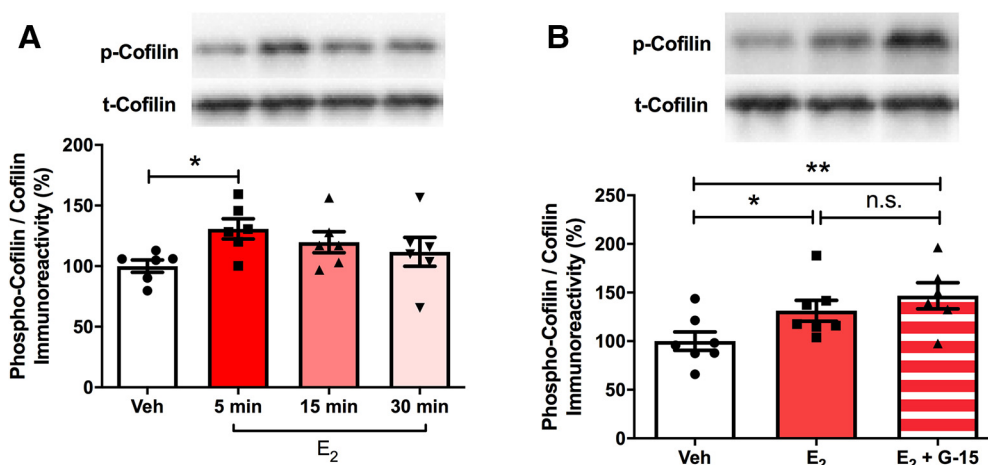


Figure 5. E₂-induced cofilin phosphorylation is not dependent on GPER activation. **A**, E₂ (5 μg/hemisphere) increased DH cofilin phosphorylation relative to vehicle (Veh) 5 min after DH infusion. **B**, ICV infusion of 10 μg E₂ significantly increased levels of phospho-cofilin in the DH. These effects were not blocked by DH infusion of G-15 (1.85 ng/hemisphere). Insets, Representative Western blots. Error bars indicate mean \pm SEM. * $p < 0.05$; ** $p < 0.01$; relative to vehicle control.

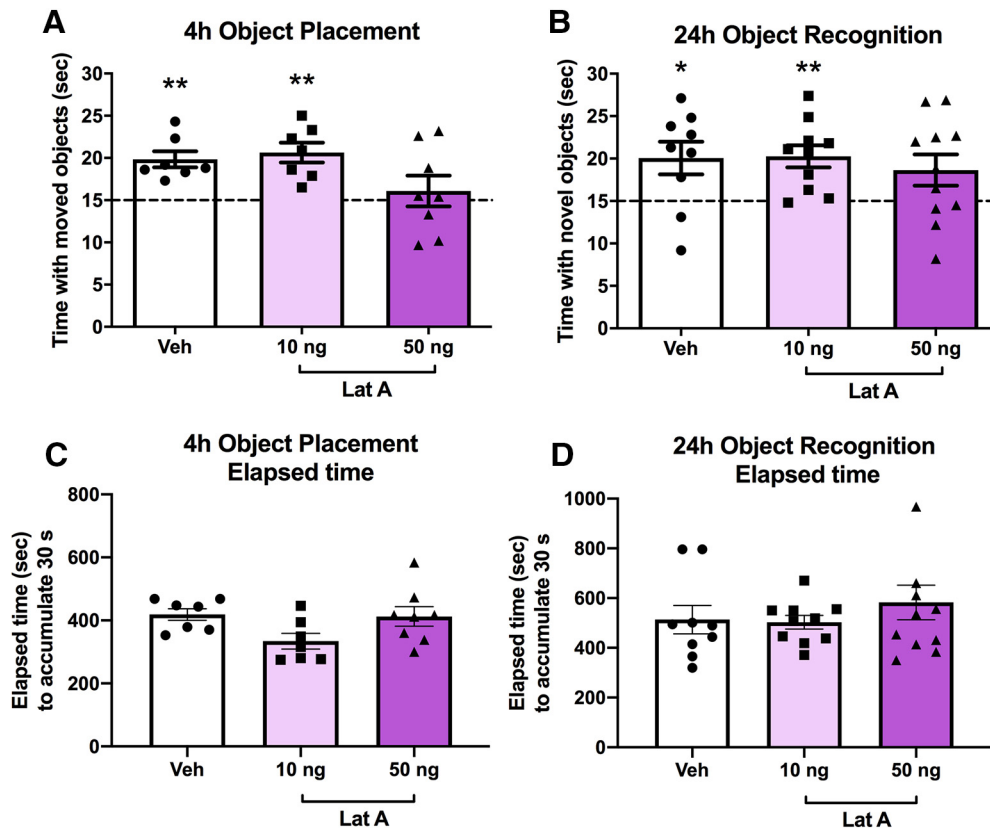


Figure 6. Actin polymerization inhibition alone impaired hippocampal memory consolidation. **A**, Mice receiving DH infusion of vehicle (Veh) or 10 ng/hemisphere latrunculin A (Lat A) spent significantly more time than chance (dashed line at 15 s) with the moved object, demonstrating intact spatial memory consolidation. In contrast, mice receiving 50 ng/hemisphere Lat A spent no more time than chance with the moved object, indicating impaired spatial memory consolidation. **B**, Similarly, the Veh and 10 ng Lat A group, but not the 50 ng Lat A group, exhibited intact object recognition memory consolidation. These data indicate a critical role for actin polymerization in hippocampal memory consolidation, and reveal a behaviorally subeffective dose of Lat A (10 ng/hemisphere) that could be used to probe the necessity of actin polymerization in G-1-induced memory enhancement. * $p < 0.05$; ** $p < 0.01$; relative to chance level of 15 s. **C**, **D**, Elapsed time to accumulate 30 s of exploration in OR and OP was not affected by Lat A infusion. Error bars indicate mean \pm SEM.

0.010), but not E_2 ($p = 0.351$), group. These results suggest that E_2 -induced cofilin phosphorylation is not dependent on GPER activation, and are consistent with our previous work showing that the effects of DH E_2 infusion on object recognition and spatial memory consolidation in OVXed mice do not depend on GPER activation (Kim et al., 2016).

GPER-mediated spine density alterations and memory enhancement are dependent on actin polymerization in the hippocampus

We next used latrunculin A to determine whether actin polymerization is involved in the memory-enhancing effects of G-1. We first tested the effects of latrunculin A alone on memory consolidation in the OR and OP tasks. Four hours after OP training, mice infused with vehicle or 10 ng, but not 50 ng, latrunculin A, spent significantly more time with the moved object than chance (vehicle, $t_{(6)} = 5.090$, $p = 0.0022$; 10 ng, $t_{(6)} = 4.814$, $p = 0.003$; 50 ng, $t_{(7)} = 0.5993$, $p = 0.5679$; Figure 6A), suggesting that 50 ng impaired spatial memory, whereas 10 ng did not. Elapsed time to accumulate 30 s of exploration did not differ among the groups ($F_{(2,19)} = 3.179$, $p = 0.0644$; Fig. 6C), suggesting no effect of latrunculin A on motivation to explore the objects. Similarly, 24 h after OR training, mice infused with vehicle or 10 ng, but not 50 ng, latrunculin A, spent significantly more time with the novel object than chance (vehicle, $t_{(8)} = 2.631$, $p = 0.0301$; 10 ng, $t_{(9)} = 4.021$, $p = 0.003$; 50 ng, $t_{(10)} = 1.991$, $p = 0.074$; Figure 6B), indicating impaired object recognition memory after treatment

with 50 ng, but not 10 ng, latrunculin A. Again, elapsed time did not differ among the OR groups ($F_{(2,27)} = 0.6382$, $p = 0.5360$; Fig. 6D). However, one-way ANOVAs conducted on time spent with the novel/moved objects were not significant for OP ($F_{(2,19)} = 3.052$, $p = 0.0709$) or OR ($F_{(2,27)} = 0.2793$, $p = 0.7585$), suggesting that both doses were generally too low to potentially impair memory consolidation. Given that mice infused with 50 ng/hemisphere did not remember object location in OP and tended to exhibit worse object identity memory in OR, a higher dose is likely to more strongly impair memory consolidation in these two tasks. Nevertheless, these experiments allowed us to determine that 10 ng/hemisphere latrunculin A had no effect on memory consolidation on its own, thereby allowing us to coinfuse this dose with G-1 in the next series of studies in which mice were infused with vehicle, G-1, or G-1 + latrunculin A.

In our first study, there was a significant main effect of treatment on CA1 apical spine density ($F_{(2,26)} = 25.67$, $p < 0.0001$; Fig. 7A), such that G-1 increased spine density relative to the vehicle ($p < 0.0001$) and G-1 + latrunculin A ($p = 0.0001$; Fig. 7A) groups, who did not differ from each other ($p = 0.9119$). Finally, we investigated a role for actin polymerization in GPER-mediated hippocampal memory enhancement. In both tasks, latrunculin A blocked the memory-enhancing effects of G-1 (Fig. 7B,C). Mice receiving G-1 + vehicle showed a significant preference for the moved object ($t_{(11)} = 3.987$, $p = 0.0021$) or novel object ($t_{(9)} = 8.073$, $p = 0.0001$), whereas mice receiving vehicle (moved object, $t_{(10)} = 0.9013$, $p = 0.3886$; novel object, $t_{(10)} =$

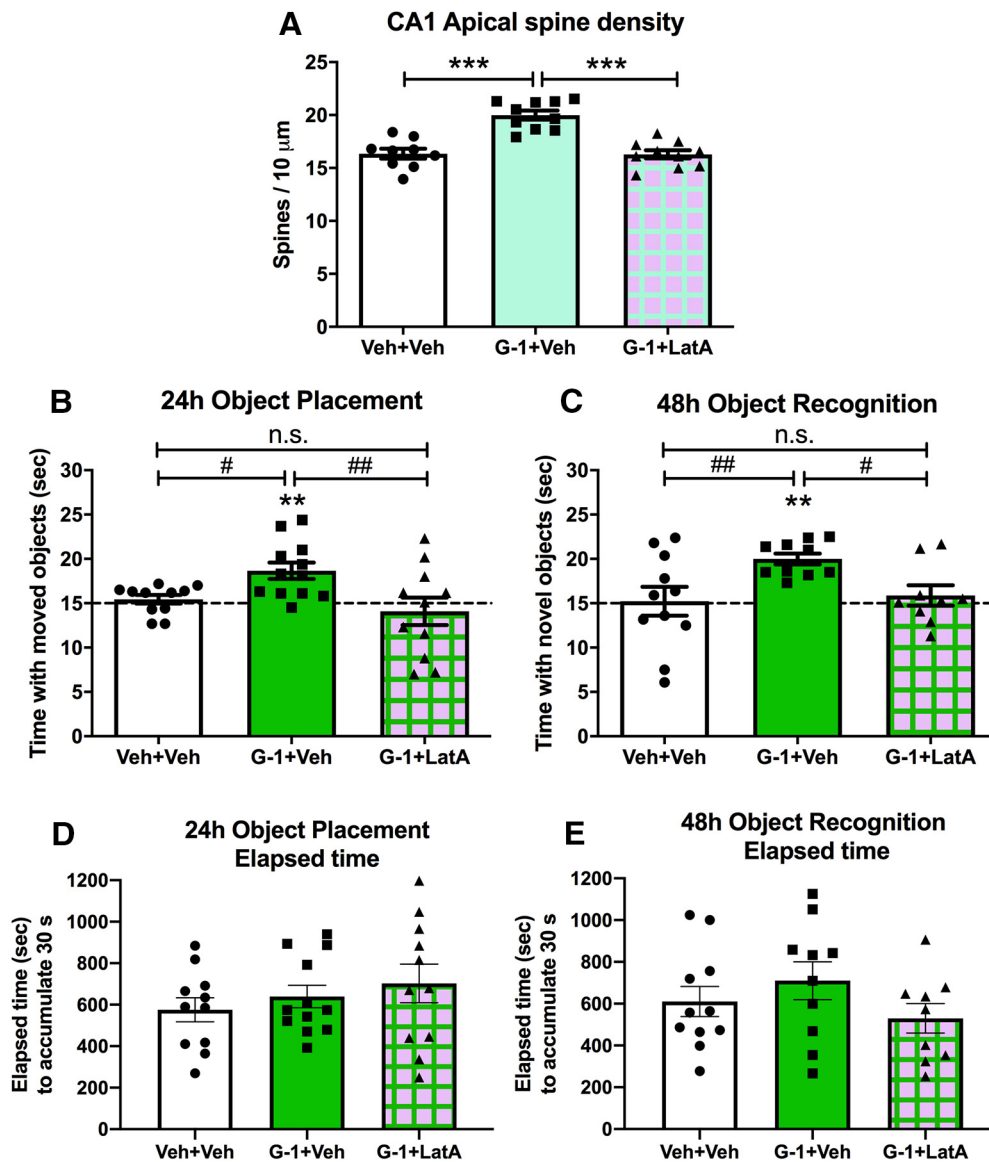


Figure 7. GPER-mediated memory enhancement and spine density alteration are dependent on hippocampal actin rearrangement. **A**, ICV infusion of 8 ng G-1 significantly increased apical spine density, and DH infusion of the behaviorally subeffective 10 ng/hemisphere latrunculin A (LatA) blocked this effect. $***p < 0.01$, relative to vehicle or G-1-treated group. **B**, Consistent with the spine data, ICV infusion of G-1 significantly enhanced spatial memory relative to Veh and chance, and DH infusion of LatA abolished this effect. **C**, Similarly, LatA prevented G-1 from enhancing object recognition memory consolidation relative to Veh and chance. $**p < 0.01$, relative to chance. $\#p < 0.05$; $\#\#p < 0.01$; relative to the Veh or G-1-treated group. **D**, **E**, In both the OP and OR tasks, elapsed time to accumulate 30 s of exploration was not affected by either dose of LatA. Error bars indicate mean \pm SEM. n.s. = not significant.

0.1463, $p = 0.8866$) or G-1 + latrunculin A did not (moved object, $t_{(10)} = 0.5915$, $p = 0.5673$; novel object, $t_{(8)} = 0.7686$, $p = 0.4642$), suggesting that actin polymerization is essential for G-1 to enhance memory. These findings were supported by significant main effects of treatment for both tasks (OP, $F_{(2,31)} = 4.935$, $p = 0.0138$; OR, $F_{(2,27)} = 4.371$, $p = 0.0227$) and *post hoc* analyses showing that the G-1 + vehicle group spent significantly more time with the moved and novel objects than the vehicle (OP, $p = 0.041$; OR, $p = 0.01$) and G-1 + latrunculin A (OP, $p = 0.005$; OR, $p = 0.031$) groups, who did not differ from each other (OP, $p = 0.3823$; OR, $p = 0.715$). Elapsed time to accumulate 30 s of exploration did not differ among the groups for either OP ($F_{(2,31)} = 0.8064$, $p = 0.4556$; Fig. 7D) or OR ($F_{(2,27)} = 1.261$, $p = 0.2995$; Fig. 7E). Together, these results suggest that GPER-mediated object recognition and spatial memory enhancement are dependent on actin polymerization in the hippocampus.

Discussion

The present findings suggest that actin polymerization plays a pivotal role in GPER-mediated memory enhancement and CA1 dendritic spine remodeling. Here, we used phosphorylation of the actin-binding protein cofilin as a proxy for actin regulation because cofilin is a critical regulator of actin dynamics, and kinase-induced inactivation of cofilin via phosphorylation is necessary for spinogenesis and LTP maintenance (Chen et al., 2007; Kramár et al., 2009). The results provide the first evidence that GPER enhances hippocampal memory consolidation and regulates dendritic spine density in females by modulating actin dynamics via JNK and cofilin signaling (Fig. 8).

We first examined whether object training could increase CA1 dendritic spine density within 40 min. Although an earlier time point than in previous studies (Moser et al., 1994; Knafo et al.,

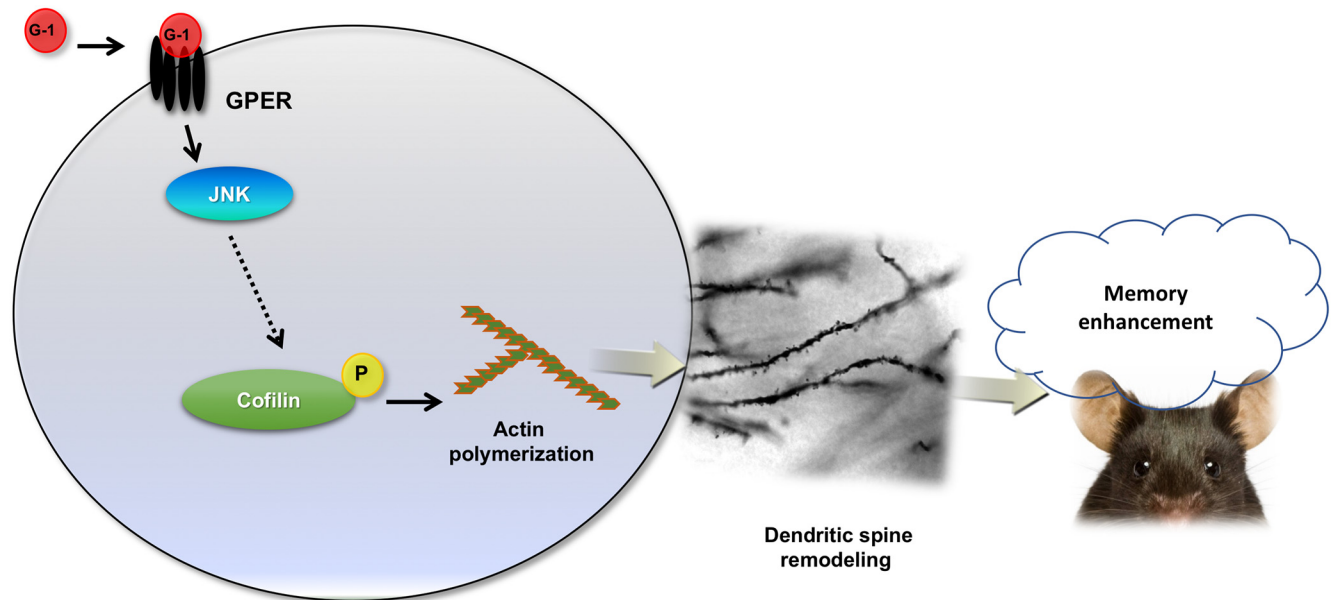


Figure 8. Proposed mechanisms involved in the role of actin polymerization in GPER-mediated hippocampal memory enhancement and spinogenesis in female mice. Hippocampal actin polymerization plays a vital role in GPER-mediated hippocampal spine density alterations and memory consolidation. Also, GPER activation increases cofilin phosphorylation in a manner that depends on JNK activation.

2001; Lai et al., 2012), we expected rapid spine remodeling because canonical Wnt/ β -catenin signaling, a key pathway involved in hippocampal spinogenesis (Murase et al., 2002; Gogolla et al., 2009; Ciani et al., 2011), is triggered in the mouse hippocampus within 5–30 min of object training (Fortress et al., 2013a). Here, training increased CA1 spine density by 11.6% and 7.5% relative to home cage and yoked controls, respectively, similar to the increase observed in the piriform cortex following olfactory learning (Knafo et al., 2001). Although density in yoked controls was statistically intermediate between the home-cage and training groups, this is to be expected, as contextual learning in the empty arena surely drives some spinogenesis. The additional 7.5% spine increase induced by object exploration, though modest, appears sufficient to support consolidation of information about both object location and identity. Previous work suggests that learning-induced increases in synapse number are transient (O'Malley et al., 1998; Nimchinsky et al., 2002); for instance, a twofold increase in hippocampal dentate spine density evident 6 h after avoidance conditioning training had subsided by 72 h (O'Malley et al., 1998). Because we examined just a single time point, the duration of the object learning-induced increase in CA1 spine density is unknown. However, these data suggest that object learning increases CA1 dendritic spine density, which may be further augmented or maintained by E_2 or G-1. Support for a multistep plasticity process comes from work in cultured cortical neurons showing that E_2 -induced spinogenesis is maintained by subsequent NMDA receptor activation (Srivastava et al., 2008).

We previously demonstrated that G-1 mimicked the beneficial effects of E_2 on object recognition and spatial memory consolidation (Kim et al., 2016), and reported that DH infusion of E_2 increases CA1 dendritic spine density in the DH (Tuscher et al., 2016). Here, we sought to determine whether GPER activation enhances memory consolidation by increasing CA1 dendritic spine density. DH G-1 infusion increased CA1 apical spine density within 40 min, which is consistent with a previous report of increased CA1 dendritic spine density 40 min after systemic G-1 treatment (Gabor et al., 2015). The timing of G-1's effects is

consistent with those of E_2 , as E_2 administered systemically or into the DH increases DH CA1 dendritic spine density 30 min and 2 h later (MacLusky et al., 2005; Inagaki et al., 2012; Tuscher et al., 2016; Avila et al., 2017). Moreover, the rapid effects of G-1 and E_2 on CA1 spine morphology are consistent as well, given the selective increase in mushroom spines produced by G-1 within 40 min (this study) and E_2 within 120 min (Avila et al., 2017). Interestingly, the effects were seen in both behaviorally naive and behaviorally trained mice. Together, these data indicate that both E_2 and GPER activation rapidly and selectively increase the density of mature spines in the dorsal hippocampal CA1.

Because we previously reported that G-1 rapidly phosphorylated both isoforms of the JNK in the DH (Kim et al., 2016), we also examined the role of JNK in GPER-mediated spinogenesis and cofilin phosphorylation. Interactions between GPER and PSD-95 have been identified in hippocampal dendritic spines (Akama et al., 2013), and JNK activity regulates PSD-95 to recruit synaptic AMPA receptors (Kim et al., 2007). Because the distribution of functional AMPA receptors is tightly correlated with dendritic spine geometry in CA1 pyramidal neurons (Matsuzaki et al., 2001), JNK signaling is likely important for synaptic transmission. We previously used the selective JNK inhibitor SP600125 to demonstrate an essential role for JNK signaling in G-1-mediated hippocampal memory consolidation in OVXed mice (Kim et al., 2016). The present data indicate that JNK signaling is also important for G-1-induced cofilin phosphorylation and CA1 dendritic spinogenesis. However, little is known about the role of JNK in mediating cofilin signaling, and data from various tissues are inconsistent. For example, JNK inhibition decreased phosphocofilin levels in vascular endothelial cells (Slee and Lowe-Krentz, 2013), and JNK activation at axon tips in hippocampal neuron cultures facilitates axon elongation by increasing cofilin phosphorylation and promoting actin polymerization (Sun et al., 2013). Although these results suggest that JNK can enhance cofilin phosphorylation, other data indicate that JNK promotes dephosphorylation of cofilin in rat aortic smooth muscle cells (Won et al., 2008). The present findings are the first to indicate that JNK

mediates the effects of hormonal modulators on cofilin phosphorylation in the brain, which suggests an important link between JNK and cofilin that is ripe for further study.

Although E_2 and other estrogenic compounds bind GPER with a high affinity in human cell lines (Funakoshi et al., 2006; Thomas and Dong, 2006), some investigators believe that GPER functions more as a collaborator in mediating the biological actions of estrogens rather than as a true estrogen receptor, such as $ER\alpha$ and $ER\beta$ (Levin, 2009; Langer et al., 2010). This notion is consistent with our previous work showing that GPER and E_2 act independently in the OVXed mouse DH to mediate OR and OP memory consolidation (Kim et al., 2016). This report showed that G-1 enhanced memory via JNK phosphorylation, whereas E_2 did so via ERK activation, and that G-15 did not prevent E_2 from enhancing memory (Kim et al., 2016). Similarly, G-15 in the present study did not prevent E_2 from increasing DH cofilin phosphorylation, indicating that GPER activation is also not required for E_2 to regulate cofilin activation. This finding is supported by other studies showing that E_2 and GPER may affect spines independently (Pedram et al., 2006; Madak-Erdogan et al., 2008; Otto et al., 2008; Briz and Baudry, 2014; Ding et al., 2015). Of the two canonical estrogen receptors, E_2 appears to rapidly regulate CA1 dendritic spines via activation of $ER\alpha$, but not $ER\beta$ (Phan et al., 2011, 2015), suggesting that E_2 effects on spine remodeling may involve activation of $ER\alpha$, but not $ER\beta$ or GPER. In addition, we showed here that E_2 -induced cofilin phosphorylation was smaller and more transient than that mediated by G-1 (Cohen's d of 1.816 for E_2 vs 2.414 for G-1). If E_2 activates cofilin by binding to GPER, then it should have produced levels of cofilin phosphorylation at least comparable with G-1 infusion. Thus, these results support the conclusion that GPER regulates cofilin-mediated actin polymerization independently of E_2 in the DH. However, we cannot yet exclude the possibility that other potential interactions between E_2 and GPER could be revealed with different drug doses or infusion timing.

Finally, to link the effects of GPER activation on cofilin and spine density with its beneficial effects on hippocampal memory consolidation, we assessed whether inhibiting actin polymerization could prevent GPER from enhancing memory. We established a dose of latrunculin A with no detrimental effects on memory consolidation to ensure that any impairment seen in combination with G-1 resulted from an interaction between the compounds rather than a general memory impairment induced by latrunculin A. DH infusion of the behaviorally subeffective dose of 10 ng/hemisphere latrunculin A prevented G-1 from increasing CA1 dendritic spine density and blocked its memory-enhancing effects, suggesting a critical role for actin polymerization in G-1-induced spine density changes and hippocampal memory enhancement. These data provide the first evidence that actin rearrangement is essential for GPER-mediated hippocampal memory consolidation. Because identifying the downstream molecular mechanisms through which GPER affects memory may assist considering GPER as a new target for the development of memory-enhancing drugs, these data may provide promising new avenues for the development of novel therapies that mimic the memory-enhancing effects of estrogens without harmful side effects.

In conclusion, this study provides novel insights into the cell-signaling mechanisms through which GPER regulates hippocampal dendritic spine density and memory consolidation. This work also supports the conclusion that hippocampal GPER does not function like a canonical estrogen receptor to regulate memory consolidation in OVXed mice, despite mimicking the beneficial effects of E_2 on spine remodeling and hippocampal memory con-

solidation. As such, these studies may suggest first steps toward new therapeutics that safely and effectively reduce memory decline in menopausal women. Although hormone therapy can reduce the risk of menopause-related memory decline and dementia in women, current treatments are not recommended because of increased risks of cancer, heart disease, and stroke (Rossouw et al., 2002; Coker et al., 2010). Because GPER influences hippocampal morphology and memory consolidation via different molecular mechanisms than E_2 , this receptor could be a valuable target for future drug development.

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