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The Effects of G-Protein-Coupled Estrogen Receptor (GPER) on Cell Signaling, Dendritic Spines, and Memory Consolidation in the Female Mouse Hippocampus

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THE EFFECTS OF G-PROTEIN-COUPLED ESTROGEN RECEPTOR (GPER) ON CELL

SIGNALING, DENDRITIC SPINES, AND MEMORY CONSOLIDATION IN THE FEMALE

MOUSE HIPPOCAMPUS

by

Jaekyoon Kim

A Dissertation Submitted in

Partial Fulfillment of the

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August 2018

ABSTRACT

THE EFFECTS OF G-PROTEIN-COUPLED ESTROGEN RECEPTOR (GPER) ON CELL
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Jaekyoon Kim

The University of Wisconsin-Milwaukee, 2018
Under the Supervision of Professor Karyn M. Frick

One of the most seminal findings in the literature on hormones and cognition is that the potent estrogen 17β-estradiol (E_2) significantly increases the density of dendritic spines on pyramidal neurons in the CA1 region of the dorsal hippocampus (DH). However, the extent to which this E_2-induced increase in hippocampal spinogenesis is necessary for memory formation remains unclear. The memory-enhancing effects of E_2 in the DH can be mediated by intracellular estrogen receptors (ERs) or by the membrane-bound ER called G-protein coupled estrogen receptor (GPER). We previously reported that infusion of a GPER agonist, G-1, into the DH of ovariectomized female mice mimicked the beneficial effects of E_2 on object recognition and spatial memory consolidation in a manner that depended on phosphorylation of the signaling kinase c-Jun N-terminal kinase (JNK). However, the role of CA1 dendritic spines in mediating GPER-induced memory consolidation, as well as the signaling mechanisms that might mediate effects of GPER activation on dendritic spine density, remain unclear. Thus, the present study examined in ovariectomized mice the effects of DH-infused G-1 on dendritic spine density and

determined whether such effects are necessary for G-1-induced memory consolidation. We first examined whether object training itself might induce increased CA1 dendritic spine density, and showed that spine density is increased by object training. Next, we found that G-1 significantly increased the density of dendritic spines on apical dendrites of CA1 pyramidal neurons in the DH. We next examined cellular mechanisms regulating G-1 induced spinogenesis by measuring effects of DH G-1 infusion of the phosphorylation of the protein cofilin, which actively regulates actin reorganization. We found that G-1 significantly increased cofilin phosphorylation in the DH, suggesting that activation of GPER may increase dendritic spine morphogenesis through actin polymerization. As with memory consolidation in our previous study, we also found that the effects of G-1 on apical CA1 spine density and cofilin phosphorylation were dependent on JNK phosphorylation in the DH. To verify the importance of actin polymerization in GPER-mediated dendritic spine morphogenesis and hippocampal memory enhancement, we applied an actin polymerization inhibitor, latrunculin A, which prevents actin polymerization and promotes filament disassembly. DH infusion of latrunculin A prevented G-1 from inducing apical CA1 spinogenesis and enhancing both object recognition and spatial memory consolidation. Collectively, these data demonstrate that GPER-mediated hippocampal memory consolidation and spine density changes are dependent on modulating actin dynamics via JNK-Cofilin signaling, supporting a critical role of actin polymerization in the GPER-induced regulation of hippocampal function in female mice.

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INTRODUCTION

The Hippocampus and Memory

The hippocampus is a bilateral limbic structure located within the medial temporal lobe. Since the famous case study of Henry Gustav Molaison, known to the world as 'Patient H.M.', the hippocampus has been one of the most researched brain regions. Initial studies of H.M.'s brain established fundamental principles of the hippocampus for memory formation (Scoville & Milner, 1957). H.M. suffered from severe epilepsy, and underwent bilateral medial temporal lobectomy surgery as an adult to remove the focus of his seizure activity. After the surgery, he experienced severely impaired declarative memory, exhibiting both anterograde amnesia and partial retrograde amnesia. In comparison, his non-declarative memory and short-term memory were preserved, showing intact short-term recognition memory for normal digit numbers and the pitch of pure tones (Corkin, 2002; Corkin, Amaral, Gonzalez, Johnson, & Hyman, 1997; Eichenbaum, 2013). This selective memory loss motivated the efforts of many investigators to better understand the neurobiological mechanisms through which medial temporal lobe structures, including the hippocampus and the adjacent perirhinal, entorhinal, and parahippocampal cortices, mediate memory formation (Squire, 2009).

Two of the most well-known functions of the hippocampus are the generation of cognitive maps for spatial navigation and mediating episodic memory processes (Smith & Mizumori, 2006). Analysis of neuronal activity in the hippocampus showed that the hippocampus is involved in mediating spatial information, as well as speed of movement, direction of movement, and match or non-match detection (Holscher, 2003). Furthermore, selective lesions of the hippocampus in animal models impair episodic memory, which refers to memory for personally experienced events (Smith & Mizumori, 2006). To examine the role of

the hippocampus in memory formation, many behavioral tests have been established for use in rodent models. For example, the Morris water maze, Barnes maze, radial arm maze, T-maze, and Y-maze are commonly used to evaluate hippocampal involvement in spatial learning and memory (Yuede, Dong, & Csernansky, 2007). A key feature of space is that it provides a context in which learning takes place, so not surprisingly, the hippocampus is also involved in contextual memory formation, as commonly tested in contextual fear conditioning (J. J. Kim & Fanselow, 1992; Lehmann, Lecluse, Houle, & Mumby, 2006). All of the aforementioned tasks use external motivation (e.g., shock, food/water restriction) to stimulate performance, yet the stress or fear associated with these manipulations may become a methodological confound.

As such, the object recognition and object location tests were developed as simple ways to assess hippocampal memory independent of externally motivating stimuli. Both tasks depend on a rodent's innate exploratory behavior, so do not involve externally applied rules, reinforcement, or punishment (Broadbent, Squire, & Clark, 2004; Ennaceur & Delacour, 1988). These tasks are also attractive because they require only brief training or habituation, and can be completed in a relatively short time. Typically, these tasks are conducted as one-trial learning tests, that is, each involves a single training trial and a single test trial for which the intertrial interval can be manipulated to measure short-term or long-term memory. Because memory of a single episode is considered more vulnerable than that based on the repetition of a reinforcer or a stimulus-response association (Antunes & Biala, 2012; Ennaceur & Delacour, 1988), these tasks can be very useful to study short- or long-term memory for an acute episodic experience.

In both tasks, subjects are placed in an arena in which they may explore 2-5 objects. During the intertrial interval, one or more objects are moved to a new location in the arena or are replaced with novel objects. The displacement of the training objects tests the subject's

knowledge of the location of objects in space, and therefore, tests spatial memory. The

introduction of novel objects into the arena tests the subject's knowledge of the identity of

training objects, and therefore, tests object recognition memory. Lesions of the hippocampus

consistently disrupt memory in the object location (aka object placement) task (Broadbent, et al.,

2004; Duva et al., 1997). Effects of hippocampal lesions on object recognition have been

inconsistent, leading to a debate about the role of the hippocampus in object recognition memory

(Broadbent, et al., 2004; Winters, Forwood, Cowell, Saksida, & Bussey, 2004). In spite of the

controversial role of the hippocampus in object recognition (Gervais, Jacob, Brake, & Mumby,

2013), more recent studies using pharmacological manipulations or chemogenetic inactivation of

the dorsal hippocampus have clearly demonstrated an important role for the dorsal portion of the

hippocampus in object recognition memory (Baker & Kim, 2002; Cohen et al., 2013; Cohen &

Stackman, 2015; Frick, Kim, Tuscher, & Fortress, 2015). One recent study showed that even

inactivation of a small portion (only about 1%) of the dorsal hippocampus can induce object

recognition memory impairment (Cohen, et al., 2013).
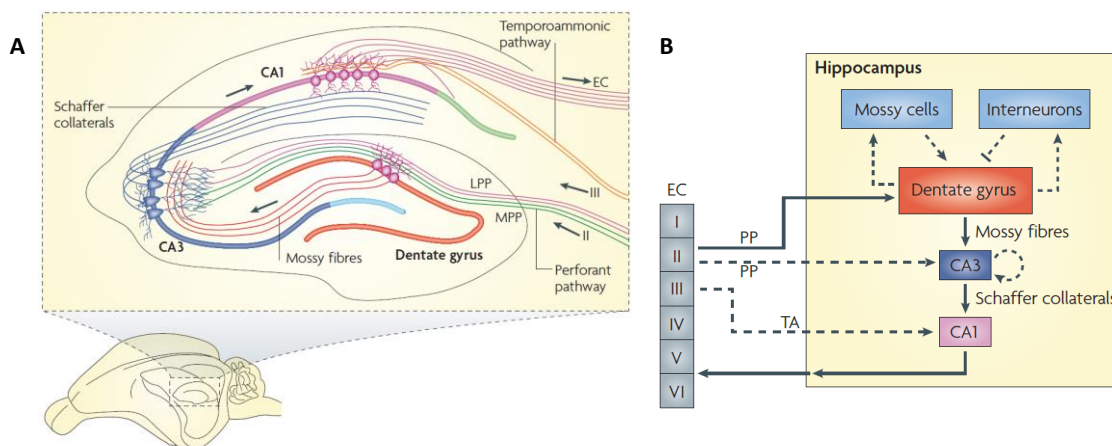
Figure 1. The neural circuitry in the rodent hippocampus. (A) An illustration of the hippocampal circuitry.
(B) Diagram of the hippocampal neural network. Solid arrows indicate the traditional excitatory trisynaptic
pathway (entorhinal cortex (EC)–dentate gyrus–CA3–CA1–EC). (A, B) Adapted from (Deng, Aimone, &
Gage, 2010).

Within the hippocampus, an elegant circuitry cooperates to facilitate learning and memory processes (Deng, Aimone, & Gage, 2010; Eichenbaum, 1996; Eichenbaum & Cohen, 1988; Morris, Garrud, Rawlins, & O'Keefe, 1982). As information from multi-modal association cortices enters the hippocampus from the adjacent entorhinal cortex layer II, it travels along a trisynaptic circuit from the granule cells of the dentate gyrus (DG) to pyramidal excitatory neurons in subregion CA3 ("Cornu Ammonis" area 3), from CA3 through CA2 to pyramidal neurons in CA1, and from CA1 to the subiculum, which then relays the information to other brain regions (Fig. 1). The trisynaptic circuit has been studied in great detail because of its simple connectivity and easily accessible characteristic structures (Stepan, Dine, & Eder, 2015). This circuit is known to play an important role in learning and memory processes (Naber, Witter, & Lopes Silva, 2000). The primary cell type within the hippocampus is the glutamatergic pyramidal neuron, which produces an action potential that excites its postsynaptic targets (Spruston, 2008). Pyramidal neurons are covered with thousands of dendritic spines which are considered a predominant site of excitatory glutamatergic synapses (von Bohlen Und Halbach, 2009). Most excitatory presynaptic terminals form synapses on spines, and it has become accepted that these dendritic spines are a primary site of synaptic plasticity (Rochefort & Konnerth, 2012). Because the goal of this dissertation is to examine the mechanisms underlying hormonal regulation of dendritic spinogenesis and memory consolidation, the next sections discuss the relationship between dendritic spines and memory, as well as the process of spinogenesis.

Dendritic Spines and Memory

Dendritic spines are small membranous protrusions from the dendritic shafts of various

types of neurons, including pyramidal neurons of the hippocampus and neocortex (Frankfurt & Luine, 2015). Dendritic spines are the smallest neuronal compartment capable of conducting neurotransmission (Shepherd, 1996). Spines are knob-like structures of various shapes and sizes with a highly plastic nature, and commonly categorized into 5 subtypes, filopodium, thin, stubby, mushroom, and cup-shaped, based on the ratio of the total length, head, and neck diameter (Maiti, Manna, Ilavazhagan, Rossignol, & Dunbar, 2015) (Fig. 2). Their morphological variations determine the strength of a synapse (Voglis & Tavernarakis, 2006). Moreover, although most spines are stable in mature neurons, certain stimuli, such as sensory input, stress, and learning, produce spine remodeling that serves specific functions (Fiala, Spacek, & Harris, 2002). Remodeled spine structures can influence synaptic connectivity and neuronal plasticity, and so dendritic spines are considered a "hot site" of synaptic plasticity (Fiala, et al., 2002; Sala & Segal, 2014). Spines are thought to play three essential roles in the nervous system: maintenance of long-term potentiation (LTP), regulation of calcium dynamics, and amplification of synaptic signals (Maiti, et al., 2015).
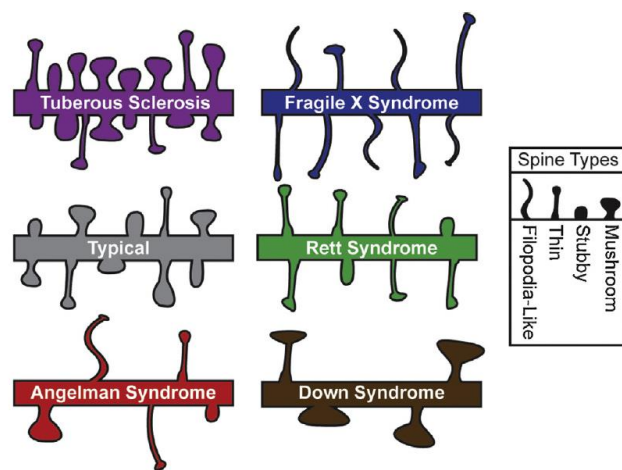
Figure 2. Characterization of dendritic spines in several neurological disorders. Morphological types of spines include thin, mushroom, and stubby, filopodia-like spines. Adapted from (Phillips & Pozzo-Miller, 2015).

Therefore, understanding dendritic spine dynamics has long attracted the attention of scientists who study neurodegenerative and psychiatric illnesses; indeed, alterations in spine number and shape have been observed in Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), autism related disorders, and Rett's syndrome (Fiala, et al., 2002; Maiti, et al., 2015; Penzes, Cahill, Jones, VanLeeuwen, & Woolfrey, 2011; Phillips & Pozzo-Miller, 2015) (Fig. 2).

Because memory formation is an adaptive process that alters neuronal connections, it is tightly linked with forms of physiological plasticity, such as long-term potentiation (LTP) and long-term depression (LTD), that are considered physiological representations of learning and memory. The degree of plasticity observed in both LTP and LTD is highly associated with dendritic spine dynamics, including spine size, number, and volume, as well as calcium signaling inside the spine (Sabatini, Maravall, & Svoboda, 2001). For instance, in hippocampal CA1 pyramidal cells, the directionality of spine alterations is associated with specific forms of synaptic plasticity: LTP is associated with increased dendritic spine size and number, whereas, LTD is associated with decreased spine number (Matsuzaki, Honkura, Ellis-Davies, & Kasai, 2004; Muller, Toni, & Buchs, 2000; Zhou, Homma, & Poo, 2004). Moreover, several studies have demonstrated that abnormalities of spine number, structure, size, or formation are associated with cognitive impairment in several neurological diseases, suggesting a strong relationship between dendritic spine and memory. For example, decreased spine density and shape abnormalities in the striatum were associated with significant cognitive and motor impairments in a mouse model of HD (Xie, Hayden, & Xu, 2010). In addition, one of the neuropathological characteristics of HD is decreased spine number in the striatum and neocortex, as well as truncated dendritic arbors and decreased spine numbers in the neocortex (Sotrel,

Williams, Kaufmann, & Myers, 1993). Moreover, decreased spine number and abnormal spine morphology are one of the early pathological alterations in AD transgenic mice (Spires et al., 2005). Recent data also showed fewer dendritic spines in the neocortex and hippocampus of AD patients than cognitively normal controls, suggesting a correlation between cognitive impairment and synaptic loss in AD (Serrano-Pozo, Frosch, Masliah, & Hyman, 2011). Several studies also have demonstrated that learning experiences mediate spine structural plasticity. For instance, fear conditioning and extinction regulate the rate of spine formation and elimination of postsynaptic dendritic spines of layer-V pyramidal neurons in the mouse frontal association cortex (Lai, Franke, & Gan, 2012). Similarly, learning to distinguish between two pairs of odors in an olfactory discrimination task increases spine density on pyramidal neurons in the rat piriform cortex (Knafo, Grossman, Barkai, & Benshalom, 2001). Spatial training also mediates an increase in spine density on basal dendrites in rat CA1 pyramidal neurons (Moser, Trommald, & Andersen, 1994). Thus, pre-clinical and clinical studies of neurodegenerative disease support the concept that dendritic spines play an important role in cognitive functions such as learning and memory. However, despite extensive investigations describing how dendritic spines are affected in patients and animal models of neurodegenerative diseases, the molecular mechanisms underlying the regulation of dendritic spines are still not fully understood.

Spine Remodeling and Actin Polymerization

One of the most important and fundamental regulators of spine morphology is the actin cytoskeleton (Penzes & 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 the function and plasticity of dendritic spines are mechanically

regulated by actin organization (Honkura, Matsuzaki, Noguchi, Ellis-Davies, & Kasai, 2008). Actin is the most abundant protein in most eukaryotic cells, and is one of the major components of the cellular scaffold maintaining cell shape. Although actin is present at both the pre- and postsynaptic terminals, it is highly enriched within dendritic spines, which constitute postsynaptic compartments (Cingolani & Goda, 2008). Actin exists in two forms: monomeric G-actin and filamentous F-actin. F-actin is an asymmetric two-stranded helical filament composed of multiple G-actin monomers (Penzes & Cahill, 2012). Actin growth is polarized with one end (the barbed end) exhibiting rapid assembly and the other end (the pointed end) losing G-actin (Cingolani & Goda, 2008). ATP-bound G-actin is added to the F-actin barbed end, and ADP-bound G-actin is removed from F-actin pointed ends (Revenu, Athman, Robine, & Louvard, 2004). Actin filaments in spine heads interact with the plasma membrane and the postsynaptic density (PSD) so that the remodeling of the actin cytoskeleton is strongly connected to synaptic function (Rochefort & Konnerth, 2012).

A variety of actin-binding proteins regulate F-actin formation and affect spine remodeling (Fig. 3). For example, profilin promotes organization of the actin cytoskeleton and is involved in the enlargement of dendritic spines during synaptic plasticity (Newey, Velamoor, Govek, & Van Aelst, 2005). Members of the Ras-like GTPase superfamily, including Rac1, cdc53, RhoA, are critical regulators of actin-binding proteins in spines; Rac1 affects the WAVE protein which directly interacts with profilin (Luo, 2002).
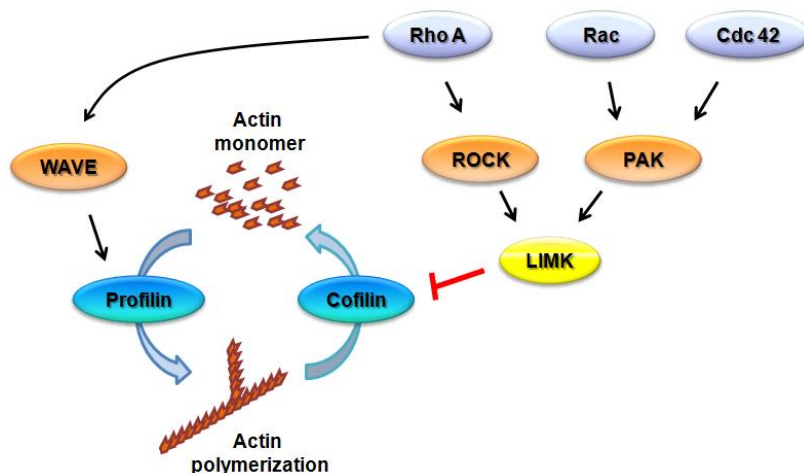
Figure 3. Schematic illustration of the molecular mechanisms of regulating actin polymerization.

In contrast to profilin, cofilin, another actin-binding protein, severs the actin cytoskeleton and promotes actin destabilization. Cofilin is considered a key regulator of actin dynamics, and inactivation of cofilin via phosphorylation by signaling kinases is necessary to increase spine volume and facilitate LTP maintenance (Babayan & Kramar, 2013; Chen, Rex, Casale, Gall, & Lynch, 2007). The binding affinity of cofilin to F-actin is controlled via phosphorylation and dephosphorylation of serine residue 3 (Rust, 2015), and LIM-kinase (LIMK) and slingshot phosphatases are believed to be potent regulators (Bernstein & Bamburg, 2010). LIMK is a well-known effecter of PAK (p21-activated kinases) (Chen, et al., 2007) and regulation of either PAK or LIMK regulates the cofilin activity mediating spine morphology alterations (Asrar et al., 2009; Meng et al., 2002). Also, Rac1 and cdc42 mediate the interaction of PAK with LIMK to phosphorylate and inactivate cofilin (Edwards, Sanders, Bokoch, & Gill, 1999; Nakayama, Harms, & Luo, 2000; Nakayama & Luo, 2000). Tyrosine kinase EphB2 and FAK (focal adhesion kinase) regulate RhoA, RhoA kinases (ROCK) and LIMK to inactivate cofilin and stabilize the mature spines (Shi, Pontrello, DeFea, Reichardt, & Ethell, 2009).

Moreover, a number of naturally occurring molecules affect actin dynamics by binding

to F-actin or G-actin, thereby, regulating actin polymerization and influencing spine remodeling. For instance, Latrunculin A, isolated from the Red Sea sponge *Negombatamagnifica*, binds to G-actin and prevents *de novo* F-actin formation, which decreases the number of spines containing GluR1, AMPAR, and NMDAR (Penzes & Cahill, 2012; Yarmola, Somasundaram, Boring, Spector, & Bubb, 2000). As such, compounds such as Latrunculin A can be used to examine the importance of actin polymerization in spine remodeling and memory formation.

Numerous modulatory factors regulate the activity of the cytoskeleton regulating pathway to influence actin polymerization. For example, the neurotrophin brain derived neurotrophic factor (BDNF) triggers actin polymerization and LTP consolidation in hippocampal slices via activation of RhoA signaling (Briz et al., 2015). BDNF itself is upregulated in the hippocampus by hormones such as 17β-estradiol (E_2; Fortress et al., 2014), which promotes hippocampal LTP *in vitro* by regulating actin polymerization (Kramar, Babayan, Gall, & Lynch, 2013). Given the well-known roles of E_2 in upregulating hippocampal dendritic spine density and promoting memory formation (Frankfurt & Luine, 2015; Tuscher, Luine, Frankfurt, & Frick, 2016), actin polymerization may be essential to both functions. Thus, the role of E_2 and estrogen receptor modulators on hippocampal dendritic spine density, actin-regulating cell signaling, and memory consolidation will be the focus of this dissertation.

Estrogen Effects on Spine Density and Underlying Mechanisms

The class of sex steroid hormones called estrogens, which include estriol, estrone (E_1), and E_2, are synthesized primarily within the gonads and placenta. However, smaller amounts of estrogens are also synthesized in non-gonadal organs such as the brain, heart, liver, bone, and muscle, and may affect many physiological processes including bone integrity, cognition, and

parenting behaviors, in addition to reproduction (Cui, Shen, & Li, 2013). E_2 is the most potent and biologically active estrogen, so it is the most common form used in biomedical research. The earliest demonstration that E_2 regulated hippocampal function came from studies in the early 1990s showing that dendritic spine density on CA1 pyramidal neurons in female rats was elevated during the estrous cycle when estrogen levels were high, and was reduced by bilateral ovariectomy, an effect that could be reversed by systemic E_2 administration (Gould, Woolley, Frankfurt, & McEwen, 1990; Woolley, Gould, Frankfurt, & McEwen, 1990). This finding has been replicated numerous times throughout the years (Hasegawa et al., 2015; Luine & Frankfurt, 2013; Phan et al., 2012; Phan et al., 2015; Tuscher, et al., 2016), with more recent studies showing that CA1 spinogenesis is induced within 30 minutes of systemic injection or dorsal hippocampal infusion of E_2 in ovariectomized rats and mice, an effect that lasts up to 4 hours (Inagaki et al., 2012; Phan et al., 2012; Tuscher et al., 2016). These effects are associated with enhanced hippocampal synaptic plasticity and memory formation (Foy et al., 1999; Inagaki, Frankfurt, & Luine, 2012; McClure, Barha, & Galea, 2013; Mukai et al., 2007; Phan, et al., 2012; Phan, et al., 2015; Woolley, Weiland, McEwen, & Schwartzkroin, 1997), suggesting a primary importance of spines to both processes. Although the molecular mechanisms regulating spine formation remain unclear, several studies discussed below suggest that actin signaling may play a role in E_2's effects on spine remodeling and synaptic potentiation.

In hippocampal slices, E_2 increases spine concentrations of F-actin and causes the induction of LTP, however, both effects are completely blocked by inhibition of actin polymerization (Kramar et al., 2009). E_2 activates the small GTPase RhoA and phosphorylates cofilin, a downstream target of RhoA; moreover, a selective inhibitor of Rho A Kinase (ROCK) completely eliminates E_2-mediated increases in EPSPs (Kramar, et al., 2009). These findings

suggest that E_2 selectively activates RhoA-ROCK-LIMK-cofilin-actin signaling in the rat hippocampus. Also, several studies indicated that inactivation of cofilin is an important step for E_2-induced spine formation (Briz & Baudry, 2014; Yuen, McEwen, & Akama, 2011). However, it is still unclear how E_2 stimulates RhoA. The McEwen group first proposed that E_2 could facilitate spine growth via the BDNF receptor tropomyosin-related kinase B (TrkB), which is known to stimulate Rho GTPase signaling, including RhoA (Spencer et al., 2008). The Lynch group then suggested that E_2 might facilitate RhoA signaling instead through β1-Integrins. These investigators showed that E_2-induced potentiation of synaptic transmission is not dependent on BDNF or TrkB signaling, but rather on β1-Integrins (Kramar, et al., 2013; W. Wang et al., 2016), suggesting that synaptic TrkB activation may be a secondary change in response to E_2-mediated synaptic effects.

Nevertheless, much more remains to be learned about the molecular mechanisms underlying E_2-induced spine remodeling in the hippocampus. For example, it is unlikely that RhoA-actin signaling is the only signaling mechanism through which E_2 regulates hippocampal dendritic spines because other signaling pathways regulate E_2-mediated spinogenesis in cultured male hippocampal neurons, including phosphoinositide 3-kinase (PI3K), protein kinase A (PKA), protein kinase C (PKC), calcium/calmodulin-dependent protein kinase II (CaMKII), extracellular signal–regulated kinase (ERK), and p38. (Hasegawa, et al., 2015; Hojo et al., 2015). Moreover, our laboratory previously showed that bilateral dorsal hippocampal infusions of the ERK inhibitor, U0126, or the mechanistic target of rapamycin (mTOR) inhibitor, rapamycin, blocked E_2-mediated CA1 spinogenesis in ovariectomized female mice, suggesting that E_2-induced spine changes in the hippocampus depend upon the activation of ERK and mTOR signaling (Tuscher, et al., 2016). This finding is of particular importance to spine remodeling because mTOR

signaling triggers local protein synthesis and is critical for hippocampal spine remodeling and memory formation in rats (Hoeffer & Klann, 2010; Sweatt, 2004). Notably, ERK-driven mTOR activation is necessary for dorsal hippocampally-infused E_2 to enhance memory consolidation in ovariectomized female mice (Fernandez et al., 2008; Fortress, Fan, Orr, Zhao, & Frick, 2013).

Effects of E_2 on Hippocampal Memory Consolidation and Cell Signaling

E_2 levels in the rat hippocampus are higher than in serum (Hojo et al., 2004), suggesting ample availability of E_2 within the hippocampus to modulate hippocampal-dependent memory. In general, estrogens enhance hippocampal memory in young and aging female rodents, as well as younger menopausal women (Duff and Hampson, 2000; Frick, 2009). In rodents, exogenous E_2 administration enhances hippocampal memory tested using a variety of tasks, including the Morris water maze, radial arm maze, and T-maze (Bimonte & Denenberg, 1999; Bohacek & Daniel, 2007; Daniel & Dohanich, 2001; Wide, Hanratty, Ting, & Galea, 2004). More recently, our lab and others have used the one-trial object recognition (OR) and object placement (OP) tasks to examine effects of E_2 on memory consolidation (Fig. 4). As described above, the hippocampus is involved in memory formation in both tasks, and both OR and OP are sensitive to several manipulations, including hormones, aging, and drug treatments (Tuscher, Fortress, Kim, & Frick, 2015). These tasks take advantage of rodent's natural instinctual motivation to explore novel stimuli. Thus, extrinsically motivating stimuli that may confound performance, such as nutrient restriction, rewards, or uncomfortable stressful environment (i.e., water submersion, electric shock, or exposure to bright light), are not necessary. Because stressors induce the release of stress hormones which can interact with estrogens (ter Horst, de Kloet, Schachinger, & Oitzl, 2012), OR and OP are particularly well suited to examine the effects of E_2

on hippocampal memory consolidation. In addition, information in these tasks can be learned within a single trial, allowing the effects of E_2 on rapid molecular mechanisms (e.g., cell signaling) to be assessed with far greater accuracy than tasks requiring multiple learning trials.
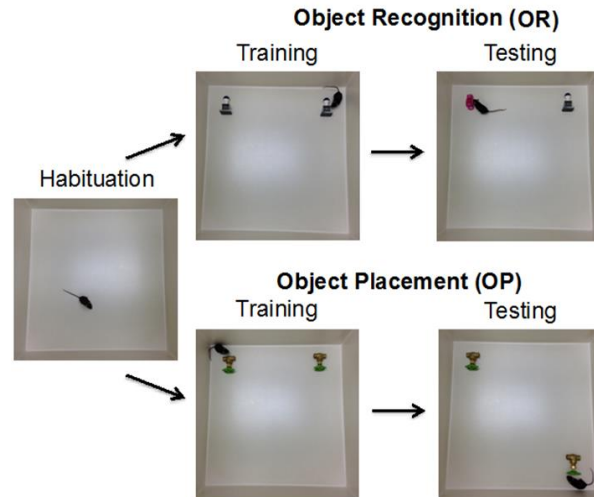
Figure 4. Overview of the object recognition and object placement task procedures.

In our laboratory, E_2 infusion is performed immediately after training in OR and OP, rather than before training, so that the effects of E_2 on memory consolidation can be pinpointed without affecting motivation, anxiety, or encoding during training. Also, because systemic treatments may affect tissues throughout the body, we infuse E_2 directly into the dorsal hippocampus to examine the role of E_2 treatment in the dorsal hippocampus specifically. In ovariectomized female mice, post-training bilateral infusion of E_2 into the dorsal hippocampus (DH) enhances hippocampal-dependent spatial memory in OP (M. I. Boulware, Heisler, & Frick, 2013; Fortress, Kim, Poole, Gould, & Frick, 2014; J. Kim, Szinte, Boulware, & Frick, 2016), as well as object recognition memory in the OR task (Fernandez, et al., 2008; Fortress, Fan, et al., 2013; Fortress, et al., 2014; J. Kim, et al., 2016; Zhao, Fan, Fortress, Boulware, & Frick, 2012; Zhao, Fan, & Frick, 2010). Because our laboratory has consistently found through the years that E_2 enhances memory in both OR and OP, we use these tasks as tools with which to identify the

molecular mechanisms through which E_2 regulates memory formation.

In female rodents, the cell-signaling mechanisms through which E_2 affects hippocampal plasticity and spinogenesis have been extensively studied. Thus far, rapid activation of ERK, PI3K, Akt, PKA, and CaMKII have been shown to play a role in E_2's effects on spines and synaptic potentiation (Fan et al., 2010; Wade & Dorsa, 2003; Wade, Robinson, Shapiro, & Dorsa, 2001; Watters, Campbell, Cunningham, Krebs, & Dorsa, 1997b; Yokomaku et al., 2003). As such, our laboratory has studied the role of many of these signaling pathways in the memory-enhancing effects of E_2. Thus far (Fig. 5), we have found that activation of p42 ERK, PI3K, PKA, and mTOR in the dorsal hippocampus within 5 minutes of DH infusion is necessary for E_2 to enhance memory consolidation in the OP and/or OR tasks (M. I. Boulware, et al., 2013; Fan, et al., 2010; Fortress, Fan, et al., 2013; Fortress, et al., 2014; Fortress, Schram, Tuscher, & Frick, 2013; Frick, Fernandez, & Harburger, 2010; Harburger, Bennett, & Frick, 2007; Lewis, Kerr, Orr, & Frick, 2008; Pechenino & Frick, 2009; Zhao, et al., 2012; Zhao, et al., 2010). In particular, we have consistently found that phosphorylation of the p42 isoform of ERK in the dorsal hippocampus is necessary for E_2 to enhance hippocampal memory (M. I. Boulware, et al., 2013; Fan, et al., 2010; Fernandez, et al., 2008; Zhao, et al., 2010). Not only does E_2 increase p42 ERK phosphorylation within 5 minutes of a DH infusion, but infusion of an ERK phosphorylation inhibitor prevents E_2 from enhancing memory consolidation, demonstrating that E_2-induced memory enhancement depends on ERK phosphorylation (Fernandez et al., 2008; Fortress et al., 2014). Furthermore, our laboratory has also shown that activation of p42 ERK is essential for specific epigenetic alterations (histone H3 acetylation) that alter the transcription of genes, like *Bdnf*, that regulate memory consolidation (Fortress, et al., 2014; Zhao, et al., 2010). Although these studies have provided some perspectives on the intracellular events underlying

the memory-enhancing effects of E_2, much more must be learned, including identifying specific estrogen receptors (ERs) involved and downstream molecular effectors.
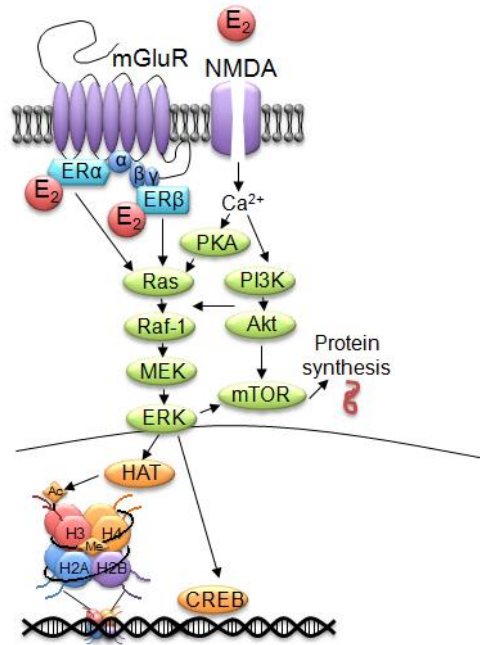
Figure 5. Schematic illustration of the molecular mechanisms required for E_2 and ERs to enhance hippocampal memory consolidation.

Estrogen Receptors

There are two general classes of ERs, intracellular (aka "classical") ERs (ERα and ERβ) and membrane ERs (e.g., GPER, ER-X). ERα and ERβ are localized in several brain regions including the hippocampus of the nuclei, dendritic spines, and axon terminals of pyramidal neurons and interneurons (T.A. Milner et al., 2005; T. A. Milner et al., 2001). When estrogens bind to ERα or ERβ in the cytoplasm, they are dimerized and move into the nucleus where they bind to estrogen response elements (ERE) to act as transcription factors to regulate gene transcription (Cheskis, Greger, Nagpal, & Freedman, 2007). Because the transcriptional effects take hours to be detected, this nuclear action of ERs, often termed a "classical" or "genomic"

mechanism, is distinctive from rapid, so-called non-classical, mechanisms mediated by membrane ERs (Hewitt, Deroo, & Korach, 2005). When estrogens bind to membrane ERs, they activate hippocampal cell-signaling cascades within minutes, suggesting an alternative mechanism of ER action.

The rapid effects of estrogens on membrane ERs were first studied using bovine serum albumin (BSA)-conjugated E_2 (BSA-E_2). BSA is a large protein, so its conjugation to E_2 prevents E_2 from passing through the cell membrane and binding intracellular ERs (Taguchi, Koslowski, & Bodenner, 2004). Interestingly, BSA-E_2 does not initiate gene transcription (Watters, Campbell, Cunningham, Krebs, & Dorsa, 1997a), yet it rapidly activates calcium signaling and ERK phosphorylation *in vitro* and *in vivo* (Carrer et al., 2003; Wu et al., 2011). In addition, our laboratory showed that dorsal hippocampal infusion of BSA-E_2 enhances OR memory consolidation in an ERK-dependent manner in ovariectomized female mice (Fernandez, et al., 2008), suggesting that membrane ER activation can influence hippocampal memory and ERK activation.

Although studies using BSA-E_2 are informative, they do not provide information about which ERs are involved and do not necessarily exclude the possible involvement of ERα and ERβ in the effects of E_2 on hippocampal memory and ERK activation. For example, two key studies showed that ERα and ERβ located at near the cell membrane interact with metabotropic glutamate receptor 1a (mGluR1a) to rapidly increase the phosphorylation of ERK and the transcription factor cAMP response element-binding protein (CREB) (M. I. Boulware, et al., 2013; M.I. Boulware et al., 2005). One of these studies demonstrated that both ERα and ERβ were present in within hippocampal detergent-resistant membranes (M. I. Boulware, et al., 2013), suggesting that ERα and ERβ localized at the membrane interact with mGluRs to initiate

the intracellular signaling in the hippocampus. In particular, mGluR1a activation was necessary

for E_2 and agonists of ERα and ERβ to trigger ERK signaling and enhance OR and OP memory

consolidation (M. I. Boulware, et al., 2013), linking membrane-associated effects of ERα and

ERβ to rapid cell signaling and memory consolidation. However, although this study supports an

essential role for ERα and ERβ with mGluR in the memory-enhancing effects of E_2, numerous

other mechanisms may also contribute to E_2-induced memory formation.

In addition to classical ERs (ERα and ERβ), E_2 may regulate memory by binding to

membrane ERs (mERs), including G-protein coupled estrogen receptor 1 (GPER), ER-X, and

Gq-mER. GPER is the most well characterized and studied mER to date, despite being only

recently classified as an mER (it was previously known as the orphan GPCR called GPR30)

(Funakoshi, Yanai, Shinoda, Kawano, & Mizukami, 2006). GPER is localized in several brain

regions, including the hippocampus (E. Brailoiu et al., 2007). Within the hippocampus, GPER is

expressed within dendritic spines of excitatory synapses and peri-synaptic regions in CA1

hippocampal neurons (Akama, Thompson, Milner, & McEwen, 2013; Srivastava & Evans,

2013). GPER is a seven transmembrane domain (7TMD) receptor including the heterotrimeric G

protein subunits Gαβγ (Filardo & Thomas, 2005), which can regulate important signaling

mechanisms like the mitogen-activated protein kinase (MAPK) ERK (Goldsmith &

Dhanasekaran, 2007). Several other downstream targets of GPER have been characterized *in

vitro*, including a SRC-like tyrosine kinase (Quinn et al., 2009), PKA via cAMP (Thomas, Pang,

Filardo, & Dong, 2005), PI3K/Akt (Maggiolini & Picard, 2010), and the Notch signaling

pathway (Ruiz-Palmero, Simon-Areces, Garcia-Segura, & Arevalo, 2011).

G-Protein Coupled Estrogen Receptor (GPER) and Hippocampal Memory

Although the role of GPER in hippocampal memory is not clearly established, some pharmacological studies have examined the role of GPER in memory processes using the selective GPER agonist, G-1, and selective antagonist, G-15 (Blasko et al., 2009; Bologa et al., 2006; Dennis et al., 2009). Chronic systemic treatment with G-1 mimics the beneficial effects of E_2 on spatial working memory in young female rats (Hammond, Mauk, Ninaci, Nelson, & Gibbs, 2009), whereas systemic treatment with G-15 impairs spatial working memory in young female rats (Hammond & Gibbs, 2011). In addition, acute systemic treatment of G-1 enhanced social recognition, object recognition, and object placement learning, and increased dendritic spine density in the CA1 region of the hippocampus (Gabor, Lymer, Phan, & Choleris, 2015). In addition, our laboratory recently provided the first evidence that GPER activation enhances hippocampal memory consolidation in young ovariectomized female mice (J. Kim, et al., 2016). We found that dorsal hippocampal infusion of G-1 enhanced (Fig. 6), whereas G-15 impaired, OR and OP memory consolidation.
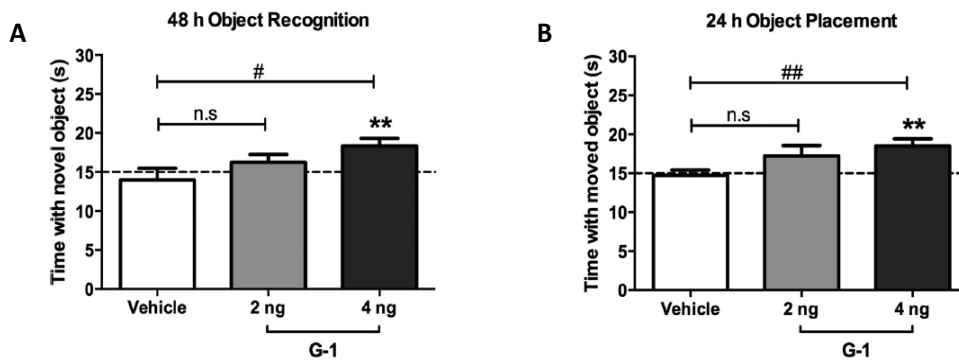
Figure 6. GPER activation enhances OP and OR memory. (A) Mice infused with 4 ng G-1, but not vehicle or 2 ng G-1, spent significantly more time with the moved object than the vehicle group or than chance 24 h after OP training, indicating enhanced spatial memory. (B) Similarly, mice receiving DH infusion of 4 ng/side G-1 (but not vehicle or 2 ng/side G-1) spent more time than chance (dashed line at 15 s) with the novel object 48 h after training. This group also spent more time with the novel object than vehicle, indicating enhanced OR memory for the familiar object (Bars represent the mean ± SEM time spent with the novel or moved object, **$p < 0.01$ relative to chance; #$p < 0.05$, ##$p < 0.01$ relative to vehicle; n.s., Non-significant).

To ensure that the effects of G-1 were specific to GPER, we confirmed that G-15 infusion blocked the effects of G-1 in OR and OP memory. We then found that E_2 enhances hippocampal memory consolidation via ERK (M. I. Boulware, et al., 2013; Fernandez, et al., 2008), whereas GPER enhances hippocampal memory consolidation by activating c-Jun N-terminal kinase (JNK) (Fig. 7) (Kim et al., 2016). To determine if the memory-enhancing effects of E_2 also required GPER, we co-infused E_2 and G-15 and found that G-15 did not block E_2's beneficial effects on memory. These findings indicated that GPER activation is not necessary for E_2 to enhance hippocampal memory consolidation (Fig. 8).
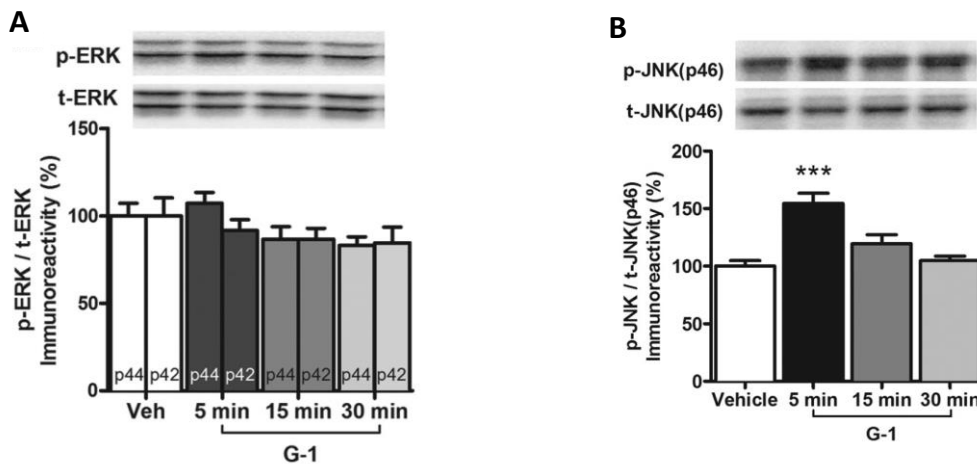
Figure 7. GPER activation increases JNK, but not ERK, phosphorylation in the DH. (A) G-1 (4 ng/side) infusion did not increase DH p42 and p44 ERK phosphorylation relative to vehicle 5, 15, or 30 minutes after DH infusion. (B) DH infusion of G-1 (4 ng/side) significantly increased phosphorylation of the JNK relative to vehicle within 5 minutes. Levels returned to baseline 15 minutes later. Insets are representative Western blots (Each Bar represents the mean ± SEM, ***$p < 0.001$ relative to vehicle control).

Although these results indicate that GPER and E_2 independently regulate memory formation, more definitive evidence came from our findings showing that E_2 enhances hippocampal memory consolidation by activating ERK, whereas GPER enhances hippocampal

memory consolidation by activating JNK (Kim et al., 2016). Indeed, ERK inhibition did not block the memory-enhancing effects of G-1, and JNK inhibition did not block the memory-enhancing effects of E_2 (Fig. 8) (Kim et al., 2016), demonstrating that E_2 and GPER influence memory via separate cell-signaling pathways, and suggesting that GPER does not function as an ER in the dorsal hippocampus.
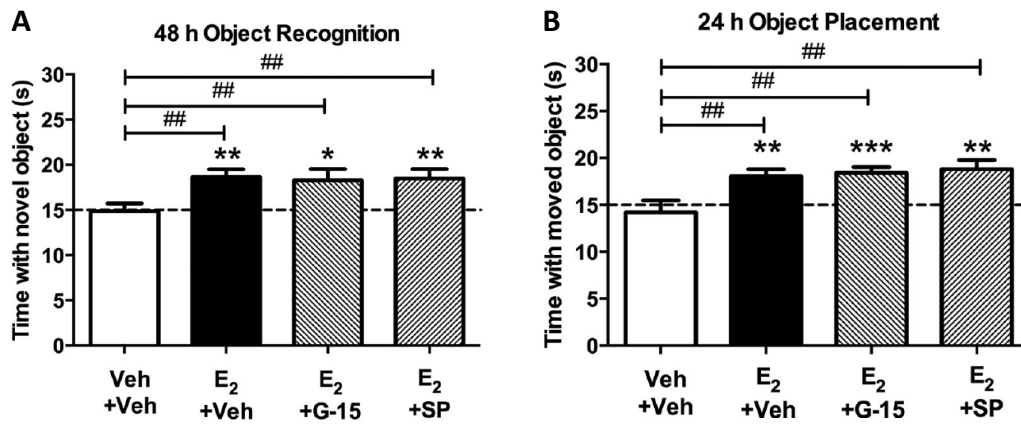
Figure 8. GPER and JNK activation in the DH are not necessary for E_2 to enhance memory. (A) Mice received DH infusion of vehicle, G-15 (1.85 ng/side), or SP600125 (2.75 ng/side) followed by ICV infusion of vehicle or E_2 (10 μg). ICV infusion of E_2 significantly enhanced OR memory relative to vehicle and chance, and these effects were not blocked by G-15 or SP600125. (B) Similarly, mice received DH and ICV infusions as described in OR. E_2 enhanced OP memory relative to vehicle and chance and the effects were not blocked by G-15 or SP600125. (Each bar represents the mean ± SEM time spent with the novel or moved object (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ relative to chance; ##$p < 0.01$ relative to vehicle).

In fact, although some data show that E_2 binds GPER with high affinity in several *in vitro* studies (E. Brailoiu, et al., 2007; Moriarty, Kim, & Bender, 2006; Prossnitz, Arterburn, & Sklar, 2007b; Revankar, Cimino, Sklar, Arterburn, & Prossnitz, 2005; Thomas, et al., 2005), some investigators insist that GPER is not a true ER, but rather has a collaborative role in regulating the biological actions of estrogens (Levin, 2009). Although somewhat unlikely in the hippocampus, given that E_2 and agonists of ERα or ERβ all regulate memory via ERK activation,

we cannot presently rule out potential interactions between GPER and ERα or ERβ in mediating G-1's effects on memory consolidation. Although it remains unclear if GPER functions as a true estrogen receptor in the hippocampus and elsewhere, data from our laboratory and others clearly demonstrate that GPER activation has similar memory-enhancing effects as E_2 in the hippocampus. These beneficial effects may provide new avenues for the future design of estrogen-based therapies to reduce the risk of age-related memory decline and Alzheimer's disease in women.

Therefore, the present study determined the mechanisms through which GPER regulates CA1 dendritic spine density and memory consolidation in the female mice hippocampus. We first examined whether object training itself might induce the increase of spine density in the CA1 region of the hippocampus. We next examined the effects of DH GPER activation on CA1 dendritic spine density and determined the extent to which GPER activation regulates the cofilin signaling pathway in the DH. We also compared the effects of G-1 and E_2 infusion on cofilin signaling and, similar to our previous findings for memory and ERK signaling, demonstrated that GPER activation is not necessary for E_2-induced phosphorylation of cofilin signaling. Finally, we examined the role of actin polymerization in GPER-mediated hippocampal memory enhancement and spinogenesis using latrunculin A, an inhibitor F-actin formation. Latrunculin A prevented G-1 from enhancing memory consolidation in the OR and OP tasks and blocked G-1's facilitation of CA1 dendritic spine density, suggesting that GPER-mediated hippocampal spine density alterations are dependent on actin rearrangement. These data demonstrate for the first time that actin polymerization is necessary for GPER to increase CA1 dendritic spine density and enhance hippocampal memory consolidation. The data also provide additional evidence that the signaling mechanisms through which GPER regulates hippocampal function in ovariectomized

female mice are independent from E_2, despite mimicking the beneficial effects of E_2 on dendritic spinogenesis and hippocampal memory consolidation.

MATERIALS AND METHODS

Subjects and Surgery. All studies used 8-12 week-old female C57BL/6 mice from Taconic. See the *Experimental Design and Statistical Analysis* section for details about the design of the experiments using the methods described below. Four days after arrival, mice were bilaterally ovariectomized (ovxed) and implanted with chronic indwelling guide cannulae into the dorsal hippocampus as previously described (M. I. Boulware, et al., 2013; Fortress, et al., 2014; J. Kim, et al., 2016). Mice were anesthetized with isoflurane gas (5% isoflurane for induction, 2% isoflurane for maintenance) on a stereotaxic apparatus (Kopf Instruments). Guide 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) were aimed at the dorsal hippocampus (1.7mm AP, 1.5 mm ML, 2.3 mm DV) or dorsal hippocampus and dorsal third ventricle (intracerebroventricular (ICV); -0.9 mm AP, ±0.0 mm ML, -2.3 mm DV). Dental cement (Darby Dental) was used to secure the guide cannulae to the skull. Mice were given six days to recover before the start of behavioral testing and drug infusion.

Drugs and Infusions. All infusions were performed at a rate of 0.5 µl/minute in the DH or 1 µl/2 minutes into the dorsal third ventricle using an infusion cannula (C3131, a 28-gauge, extending 0.8 mm beyond the 1.5 mm guide for DH or C313I-SPC, a 28-gauge, extending 1 mm beyond the 1.8 mm guide for dorsal third ventricle). Infusions were controlled by a microinfusion pump (KDS Legato 180, KD Scientific) attached to a 10 µl Hamilton syringe. The syringe was connected to the infusion cannula with PE20 polyethylene tubing. Each infusion was followed by a one-minute waiting period to allow the drug to diffuse through the tissue and prevent diffusion back up the cannula track. G-1, 1-[4-(6-bromobenzo[1,3]dioxol-5yl)-3a,4,5,9b-tetrahydro-3H-

cyclopenta [c]quinolin-8-yl]-ethanone (Sandia Biotech), was dissolved in 16% DMSO in 0.9%

saline and infused at a dose of 4 ng/side into the DH or 8 ng ICV (J. Kim, et al., 2016). The

vehicle control for G-1 was 16% DMSO in 0.9% saline. G-15, (3aS*,4R*,9bR*)-4-(6-Bromo-

1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinoline (Sandia Biotech), was dissolved in

2% DMSO and infused at a dose of 1.85 ng/side into the DH (J. Kim, et al., 2016). In our

previous work, we showed that this dose of G-15 does not affect memory on its own, but does

block the memory-enhancing effects of G-1 (J. Kim, et al., 2016). The vehicle control for G-15

was 2% DMSO in 0.9% saline. 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/side into the DH (J.

Kim, et al., 2016). SP600125 is a selective inhibitor for JNK that does not affect ERK and p38 at

concentrations below 10 μM (Bennett et al., 2001). In our previous work, we found that 2.75

ng/side SP600125 blocks the memory-enhancing effects of G-1 but has no effect on memory on

its own (J. Kim, et al., 2016). Cyclodextrin-encapsulated E_2 (Sigma-Aldrich) was dissolved in

0.9% saline and infused at doses of 5 μg/side into the DH or 10 μg ICV as previous studies (M. I.

Boulware, et al., 2013; Fernandez, et al., 2008; J. Kim, et al., 2016). The vehicle control for E_2

was 2-hydroxypropyl-β-cyclodextrin (HBC, Sigma-Aldrich), dissolved in 0.9% saline.

Latrunculin A (Enzo Life Sciences) was dissolved in 1% DMSO in saline and infused into the

DH at doses of 10 or 50 ng/side, based on previous *in vitro* and *iv vivo* studies (Li et al., 2015;

Nelson, Witty, Williamson, & Daniel, 2012; Yarmola, et al., 2000; 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. Rapid Golgi Stain Kit (FD

NeuroTechnologies) was used for Golgi staining as described previously (Tuscher et al., 2016). The Golgi staining technique is a simple histological procedure, in which brain tissue is exposed to potassium dichromate and impregnated with heavy metal ions such as silver and mercury (Torres-Fernandez, 2006) (Mancuso, Chen, Li, Xue, & Wong, 2013). This stain labels all but the nucleus and mitochondria of a few select neurons such that it reveals a complete three-dimensional neuron morphology of a subset of neurons, thus, making individual spines detectable. Brain tissue was immersed in the impregnation solution, containing mercuric chloride, potassium dichromate, and potassium chromate, at room temperature in the dark for two weeks. The solution was replaced 24 hours after the tissue first immersed. Then the tissue was transferred into a third solution for 48 hours and the solution was replaced 24 hours after the tissue transferred. The tissue was sliced into 100 µm thick sections using a cryostat at -30 °C and mounted on gelatin-coated microscope slides. The sections were stained using the developing solution and coversliped with Permount solution. Golgi-stained sections were covered by foil to protect from light whenever possible.

Dendritic Spine Counting. Dendritic spines were counted under an Olympus BX51WI microscope (100x with oil) using NeuroLucida (v11.08). Tertiary apical dendrites were selected from pyramidal neurons in the CA1 region of the hippocampus, based on previously established studies that showed E_2 infusion increases spine density in the CA1 region (Frankfurt, Salas-Ramirez, Friedman, & Luine, 2011; Tuscher, et al., 2016). The selected dendrites were limited to those 10-20 µm long and 0.5-1.3 µm thick. At least six neurons per brain were selected and 2-3 dendrites were selected per neuron. Thus, a total of at least 12 segments per brain were counted. Spine density was presented as the number of spines/10 µm dendrite.

Western blotting. Five, 15, or 30 minutes after infusion, mice were cervically dislocated and decapitated, and the dorsal hippocampus 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 µl/mg in lysis buffer and homogenized by sonication (Branson Sonifier 250). Homogenates were then electrophoresed on 10% Tris-HCl precast gels (Bio-Rad) and transferred to a nitrocellulose membrane. Western blots were blocked with 5% skim milk and incubated with an anti-phospho-cofilin (1:1000; Cell Signaling Technology) primary antibody overnight. The blots were then incubated with the appropriate HRP-conjugated secondary antibody (1:5,000; Cell Signaling), and developed using West Dura chemiluminescent substrate (Pierce). A ChemiDocMP gel imager (Bio-Rad) was used for signal detection of protein expression, and accompanying analysis/quantification software, Image Lab (Bio-Rad), was used to perform densitometry. Blots were then stripped with 0.2M NaOH, incubated with an antibody for total cofilin (1:1000; Cell Signaling Technology), and quantified for normalization of phospho-cofilin to total cofilin.

Object Recognition and Object Placement. OR and OP were performed as described previously (M. I. Boulware, et al., 2013; Fernandez, et al., 2008; J. Kim, et al., 2016). The order of OR and OP testing were 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 seconds/day) for 3 days to habituate them to the experimenters. Mice were then habituated in an empty white arena (width, 60 cm; length, 60 cm; height, 47 cm) for 5 minutes/day for two days. On the training day for each task,

two identical objects were placed near the upper-right and upper-left corners of the arena. Mice remained in the arena until they had accumulated a total of 30 sec exploring the objects (indicated when the mouse's nose or whiskers were directed at or in contact with 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). Because mice inherently prefer novelty, mice that remember the identity or location of the training objects spend more time than chance with the novel or moved objects. Chance is set at 15 seconds because this value indicates that mice spend equal amounts of time with each object. For OR, a 24-hour delay was used to test the memory-impairing effects of latrunculin A and a 48-hour delay was used to test the memory-enhancing effects of G-1 because young ovariectomized vehicle-infused female mice remember the training objects after 24 h, but not 48 h (M. I. Boulware, et al., 2013; Fernandez, et al., 2008; J. Kim, et al., 2016). For OP, we used a 4-hour delay to test the memory impairing effects of latrunculin A and the 24-hour delay to test memory enhancing effects of G-1, based on previously established studies that showed vehicle-infused female mice remember object locations after 4, but not in 24, hours (M. I. Boulware, et al., 2013; Fernandez, et al., 2008; J. 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, investigation time for the objects and elapsed time were recorded using ANYmaze tracking software (Stoelting).

Experimental Design and Statistical Analysis

Experiment 1. This experiment examined whether object training itself regulates spine density in the CA1 region of the hippocampus among ovxed female mice. One week after surgery, a subset

of ovxed mice underwent habituation and object training with two identical objects, followed 40 minutes later by brain tissue collection for Golgi staining. These object trained mice (n = 9) were compared with home cage controls (n = 8) and no-training controls (n = 11). No-training 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 box for the average duration as the trained mice (8 minutes). Home cage controls remained in their home cages for the duration of the study. Object trained and no-training mice were killed 40 minutes after training to examine learning-induced changes in CA1 apical spine density.

Experiment 2a and 2b. Experiment 2a tested effects of DH G-1 infusion on dendritic spine density and cofilin phosphorylation. This experiment required two sets of ovxed mice. The first received vehicle (n = 5) or G-1 (n = 5) infusion and their brain tissue collected 40 minutes later for Golgi spine analysis as described above. The second was killed 5 (n = 5), 15 (n = 5), or 30 minutes (n = 5) after G-1 infusion and compared with vehicle (n = 5) infused mice killed at each time point. The dorsal hippocampus was dissected immediately for Western blot analysis of cofilin phosphorylation. Experiment 2b examined whether GPER activation or JNK cell signaling are necessary for the G-1-induced alterations in cofilin phosphorylation and spine density changes observed in Experiment 2a. The antagonist GPER G-15 was used to test GPER activation and the JNK inhibitor SP600125 was used to examine 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 Golgi spine analysis 40 minutes 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 + SP60012 (n

= 7), and dorsal hippocampal tissue was collected 5 minutes 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 phosphorylation of cofilin is dependent on GPER activation. To establish effects of E_2 on cofilin phosphorylation, ovxed mice were killed 5 (n = 6), 15 (n = 6), or 30 minutes (n = 6) after E_2 infusion and compared with vehicle (n = 6) infused mice killed at each time point. Dorsal hippocampal tissue was collected for Western blot analysis of cofilin phosphorylation. To determine if GPER activation is necessary 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 dorsal hippocampus was dissected 5 min later for Western blot analysis of cofilin phosphorylation.

Experiment 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 latruculin A, 10 ng/side (OP; n = 7, OR; n =10) or 50 ng/side (OP; n = 8, OR; n =11). Experiment 4b. examined whether actin polymerization is necessary for GPER-mediated hippocampal spine density alterations and memory enhancement. 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 were tested 48 and 24 hours later, respectively, as described above. 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 minutes later. Whole brains were collected, and Golgi impregnated as described above.

Statistical Analysis. Statistical analyses were conducted in GraphPad Prism 6 (La Jolla, CA). Dendritic spine and Western blot data in all studies were analyzed with one-way ANOVA followed by Fisher's LSD post-hoc tests or *t*-tests to examine treatment effects and between-group differences. For the OR and OP tasks, one-sample *t*-tests were used to determine whether the time spent with each object significantly differed from chance (15 seconds), showing evidence of learning. To examine treatment differences among groups, one-way ANOVAs followed by Fisher's LSD post-hoc tests were used. Statistical significance was determined as $p \leq 0.05$.

RESULTS

Dendritic Spine Density in the Hippocampus is Altered by Object Training

As the primary site of synaptic input to neurons, dendritic spines are assumed to play an essential role in learning memory. However, although several studies have link changes in dendritic spine morphology with learning (Knafo, et al., 2001; Moser, et al., 1994; Nimchinsky, Sabatini, & Svoboda, 2002; O'Malley, O'Connell, Murphy, & Regan, 2000), surprisingly little is known about learning affects CA1 dendritic spine density, including object learning. Previous results from our own laboratory showed that DH infusion of E_2 rapidly increases CA1 dendritic spine density via the same cell signaling pathways necessary for E_2 to enhance object memory consolidation (Tuscher, et al., 2016), suggesting that object training itself might regulate dendritic spine density. Therefore, we sought to determine if object training influences CA1 dendritic spine density in ovxed mice. Mice were habituated and trained with two identical objects as described above and then whole brains were collected 40 minutes later and Golgi impregnated. As illustrated in Fig. 9C, the density of apical spines on CA1 tertiary dendrites in object trained mice was significantly higher than that of the home cage control, as indicated by a main effects of training ($F_{(2,25)} = 3.507$, $p = 0.0454$) and group difference ($p < 0.05$) between the object training and home cage groups. Spine density in the no-training control group (empty box) was not statistically different from that of home cage controls, suggesting that the increase seen in the object training group was associated with object exploration.
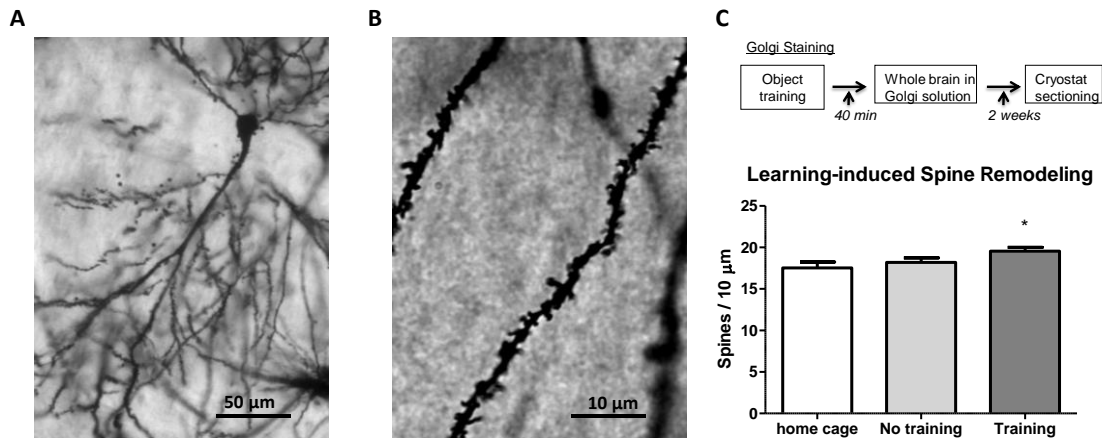
Figure 9. Learning-induced changes in tertiary CA1 apical spine density. (A, B) Photomicrograph of Golgi-impregnated CA1 pyramidal neurons under 20X (A) and under oil 100X (B) (C) Forty minutes after training, CA1 apical spine density was significantly increased in the object training group only, suggesting that the increased CA1 apical spine density is induced by object training (Bars represent the mean ± SEM, *$p < 0.05$ relative to home cage controls).

GPER Activation Regulates CA1 Dendritic Spine Density and Hippocampal Cofilin Phosphorylation in a JNK-dependent Manner

Previously, we demonstrated that DH infusion of the GPER agonist, G-1, mimicked the beneficial effects of E_2 on object recognition and spatial memory consolidation in ovxed female mice (J. Kim, et al., 2016). We also reported that DH infusion of E_2 increases dendritic spine density in the DH within just 30 minutes (Tuscher, et al., 2016). Recently, one study reported that pre-training systemic G-1 treatment increases CA1 apical dendritic spine density and facilitates object and spatial learning and memory (Gabor, et al., 2015). However, systemic injection of G-1 may cause physiological changes on other brain regions as well as the dorsal hippocampus that could account for its effects on memory. Moreover, little is known about the cellular mechanisms underlying the effects of GPER activation on spinogenesis in the hippocampus. Thus, we first examined the effects of bilateral DH G-1 infusion on CA1 dendritic spine density. Mice received

bilateral DH infusion of vehicle or G-1 (4 ng/side) and then whole brains were collected 40 minutes later. This time point was selected based on a previous study that showed increased CA1 dendritic spine density 40 minutes after systemic G-1 treatment (Gabor, et al., 2015) and was within the 30 minutes to 2 hours time window in which we had previously found that DH E_2 infusion increased CA1 dendritic spine density (Tuscher, et al., 2016). Analysis of Golgi-stained tissue revealed that DH infusion of G-1 significantly increased CA1 dendritic spine density relative to vehicle 40 minutes after infusion ($t_{(8)} = 3.056$, $p = 0.0157$; Fig. 10A).

Next, we examined the effects of G-1 on cofilin phosphorylation. A new set of mice received bilateral DH infusions of vehicle or G-1 (4 ng/side) and then DH tissue was dissected for Western blotting at three time points (5, 15, 30 minutes). These time points were selected based on our previous work demonstrating that DH infusion of G-1 increases phosphorylation of JNK 5, but not 15 or 30, minutes after infusion (J. Kim, et al., 2016). Here, the main effect of treatment was not significant ($F_{(3,16)} = 2.552$, $p = 0.0921$), but a priori t-tests indicated a significant increase in cofilin phosphorylation following G-1 treatment relative to vehicle 5 and 15 minutes after infusion (5 minutes, $t_{(8)} = 3.818$, $p = 0.0051$; 15 minutes, $t_{(8)} = 2.311$, $p = 0.0496$; Fig. 10B). Phospho-cofilin levels returned to baseline 30 minutes after G-1 infusion. These data suggest that GPER may affect hippocampal dendritic spine density via cofilin signaling.
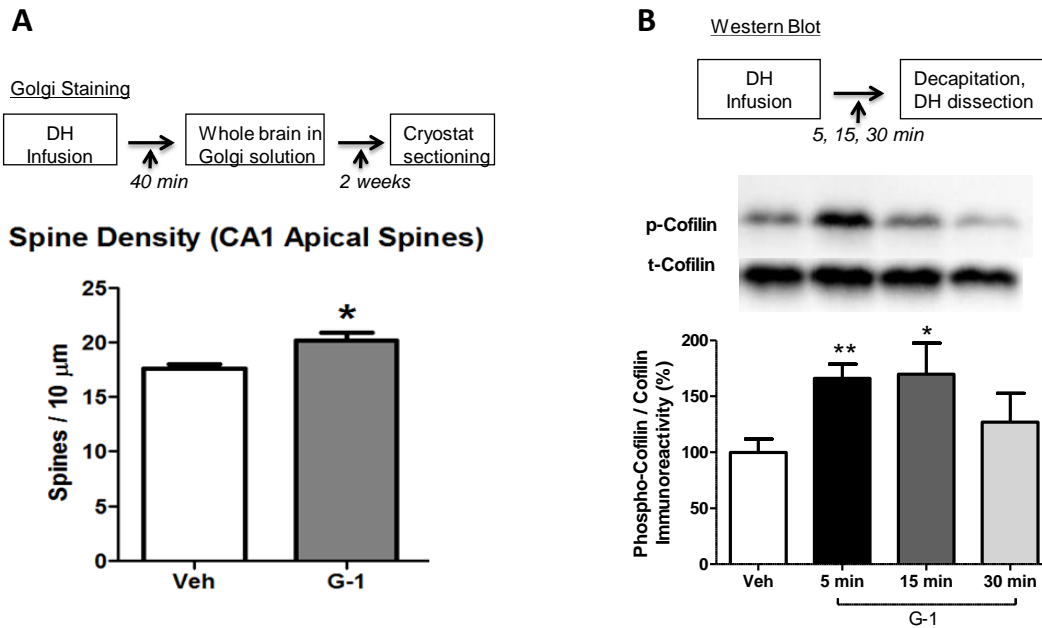
Figure 10. G-1-induced dendritic spine density changes and cofilin phosphorylation. (A) DH infusion of 4 ng/side G-1 into the dorsal hippocampus increased apical spine density relative to vehicle 40 minutes later. (B) G-1 (4 ng/side) infusion increased DH cofilin phosphorylation relative to vehicle 5 and 15 minutes after DH infusion (Bars represent the mean ± SEM, $*p < 0.05$, $**p < 0.01$ relative to vehicle control).

To confirm that the G-1-induced phosphorylation of cofilin occurs via GPER activation, we co-infused G-1 with G-15, a selective GPER antagonist. Vehicle or G-1 (8 ng) was infused ICV and vehicle or G-15 (1.85 ng/side) was infused bilaterally into the DH. Based on the data from Fig. 10, mice were cervically dislocated and decapitated for collection of DH tissue 5 minutes after infusion. Cofilin phosphorylation was influenced by G-1 and G-15 treatment, as indicated by a significant main effect of treatment ($F_{(2,14)} = 13.83$, $p = 0.005$; Fig. 11A). *Post hoc* tests showed that levels of phospho-cofilin were significantly higher than vehicle in the G-1 group ($p < 0.01$), but not the G-1 + G-15 group.

In addition, to determine the importance of JNK signaling in GPER-mediated cofilin phosphorylation, we co-infused G-1 with SP600125, a selective JNK inhibitor. Mice received

DH infusion of vehicle or SP600125 (2.75 ng/side) plus ICV infusion of vehicle or G-1 (8 ng);

DH tissue was collected 5 minutes later. Consistent with the effects of DH G-1 infusion, ICV

infusion of G-1 increased DH cofilin phosphorylation, and infusion of SP600125 into the DH

completely blocked these effects ($F_{(2,19)}$ = 5.031, p = 0.0148; Fig. 11B). *Post hoc* tests indicated

that only the G-1 infusion group exhibited significantly higher phospho-cofilin levels than

vehicle ($p < 0.05$), indicating that SP600125 blocked the effects of G-1 on cofilin
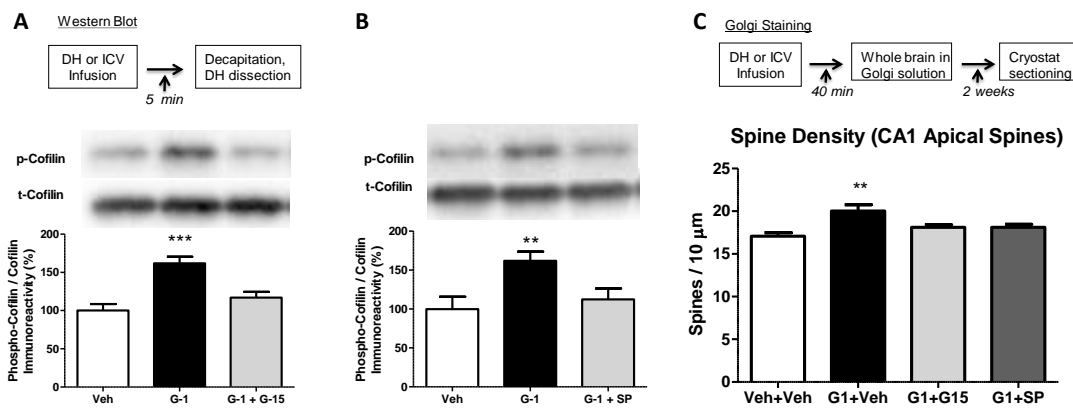
phosphorylation.

Figure 11. G-1-induced cofilin phosphorylation and spine density changes are dependent on GPER activation and JNK signaling. (A) ICV infusion of 8 ng G-1 significantly increased levels of phospho-cofilin relative to vehicle. These effects were blocked by DH infusion of G-15 (1.85 ng/side). (B) Increased levels of phospho-cofilin by ICV infusion of 8 ng G-1 were blocked by DH infusion of SP600125 (2.75 ng/side). Insets are representative Western blots. (C) ICV infusion of 8 ng G-1 significantly increased CA1 apical spine density relative to vehicle 40 minutes after infusion. DH infusion of either G-15 (1.85 ng/side) or SP600125 (2.75 ng/side) blocked G-1 infusion effects on CA1 apical spine density (Bars represent the mean ± SEM, *$p < 0.05$, **$p < 0.01$ relative to vehicle control).

Finally, we next investigated the importance of GPER activation and JNK signaling in

G-1-mediated dendritic spine density alterations. Mice were ovariectomized, implanted with

cannulae, and infused with vehicle + vehicle, G-1 + vehicle, G-1 + G-15, or G-1 + SP600125,

and then 40 minutes after infusion, mice were cervically dislocated and the whole brain collected

for Golgi analysis. Spine counting analyses showed that drug treatment altered dendritic spine

density ($F_{(3,39)}$ = 6.680, p = 0.001; Fig. 11C). ICV infusion of G-1 significantly increased CA1

apical spine density relative to vehicle ($p < 0.01$), and DH infusion of either G-15 or SP600125

blocked this effect (Fig. 11C), suggesting that G-1-induced spine density changes are also

dependent on GPER activation and JNK signaling.

Together, these three studies demonstrate that the ability of G-1 activation to increase

cofilin phosphorylation and CA1 dendritic spine density depends 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 (J.

Kim, et al., 2016). Collectively the data suggest that GPER activation regulates CA1 spine

density and memory consolidation via JNK- and cofilin-regulated alterations in actin

polymerization.

GPER Activation is Not Necessary for E$_2$-induced Cofilin Phosphorylation

E$_2$ significantly increases cofilin phosphorylation in rat hippocampal slices (Kramar, et

al., 2009), but little is known about mechanisms underlying E$_2$- or estrogen receptor-induced

changes in actin polymerization *in vivo*. Although it has been suggested that ERα and ERβ play a

role in cofilin-related actin polymerization signaling (Briz & 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 (J. Kim, et al., 2016), we examined both the effects of DH

E$_2$ infusion on cofilin phosphorylation and whether GPER activation is necessary for E$_2$-

mediated cofilin signaling alterations in the DH. Ovxed mice received bilateral DH infusions of

vehicle or E$_2$ (5 μg/side) and then DH tissue was dissected for Western blotting at 5, 15, and 30

minutes. These time points were selected based on the data in Fig. 8B, as well as effects of DH

E_2 infusion on phosphorylation of the p42 isoform ERK 5 minutes after infusion (M. I.

Boulware, et al., 2013; Fernandez, et al., 2008; J. Kim, et al., 2016). The main effect of treatment

was not significant ($F_{(3,20)}$ = 2.149, p = 0.1259), but an a priori t-test revealed that E_2 infusion

increased cofilin phosphorylation relative to vehicle 5 minutes after infusion ($t_{(10)}$ = 3.148, p =

0.0104; Fig. 12A). Phospho-cofilin levels were not significantly different from vehicle 15 and 30

minutes after E_2 infusion. These data suggest that E_2 rapidly and transiently increases cofilin

signaling in the DH.
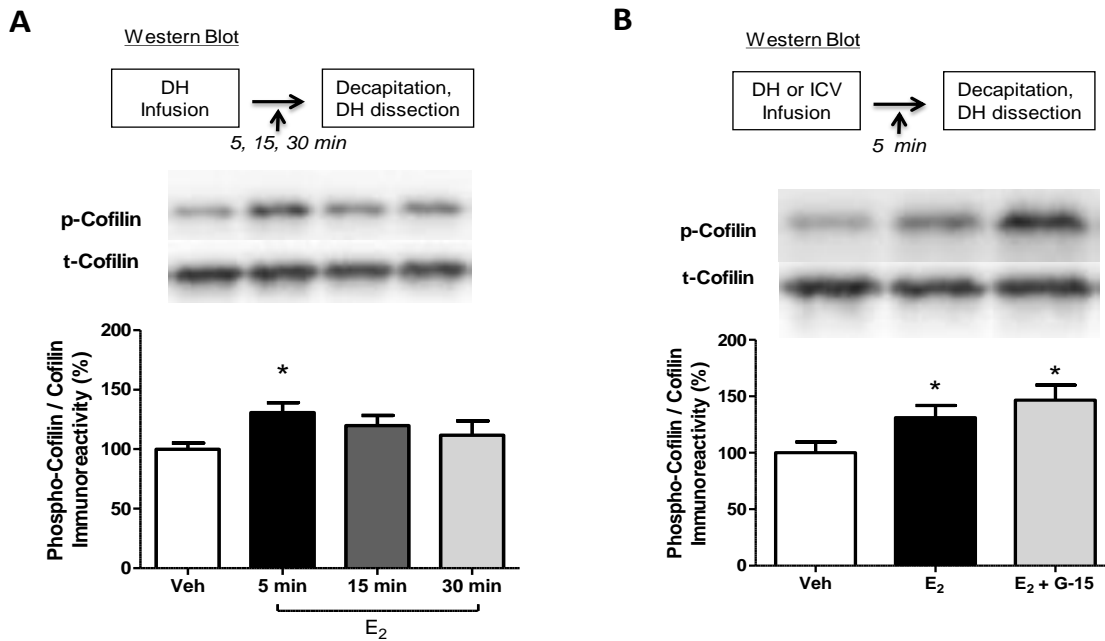
Figure 12. E_2-induced cofilin phosphorylation is not dependent on GPER activation. (A) E_2 (5 µg/side) increased DH cofilin phosphorylation relative to vehicle 5 minutes after DH infusion. (B) ICV infusion of 10 µg E_2 significantly increased levels of phospho-cofilin. These effects were not blocked by DH infusion of G-15 (1.85 ng/side). Insets are representative Western blots (Bars represent the mean ± SEM, *p < 0.05 relative to vehicle control).

To test whether activation of GPER is necessary for E_2-induced phosphorylation of

cofilin, we co-infused E_2 with G-15. Five minutes after infusion, mice were cervically dislocated

and decapitated for DH tissue collection. The main effect of treatment was significant ($F_{(2,17)}$ =

4.499, $p = 0.027$; Fig. 12B), indicating that E_2 treatment altered cofilin phosphorylation. ICV E_2 infusion increased cofilin phosphorylation relative to vehicle ($p < 0.05$) and G-15 did not this effect ($p < 0.05$), suggesting E_2-induced cofilin phosphorylation is not dependent on GPER activation. These data 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 (J. Kim, et al., 2016).

GPER-mediated Spine Density Alterations and Memory Enhancement are Dependent on Actin Polymerization in the Hippocampus

Spine remodeling can influence synaptic connectivity and neuronal plasticity (Fiala, et al., 2002). Because neuronal plasticity is tightly connected with the process of memory formation (Takeuchi, Duszkiewicz, & Morris, 2014), and dendritic spine dynamics are highly associated with neuronal plasticity (Sala & Segal, 2014), dendritic spines are thought to play an important role in learning and memory. The function and plasticity of dendritic spines are mechanically regulated by actin structure in hippocampal synapses (Honkura, et al., 2008). As actin polymerization is one the most important mechanisms in the regulation of spine development and motility, the role of actin polymerization in the formation or enlargement of dendritic spines and memory formation has been examined (Penzes & Cahill, 2012) using latrunculin A, an inhibitor of *de novo* F-actin formation. Latrunculin A is a natural toxin purified from the red sea sponge *Latrunculia magnifica,* and binds at the ATP binding site of G-actin to prevent *de novo* actin polymerization and promote filament disassembly (Yarmola, et al., 2000). Several studies have used latrunculin A to investigate the functional role of actin polymerization in synaptic efficacy and memory, such as postsynaptic AMPA receptor trafficking, object placement

memory, and drug-associated memory (Li, et al., 2015; Nelson, et al., 2012; Young, et al., 2014; Zhou, Xiao, & Nicoll, 2001). Intrahippocampal infusion of latrunculin A in the rat blocks object placement memory (Nelson, et al., 2012), suggesting the feasibility of using latrunculin A to test the role of actin polymerization in memory in the mouse hippocampus.
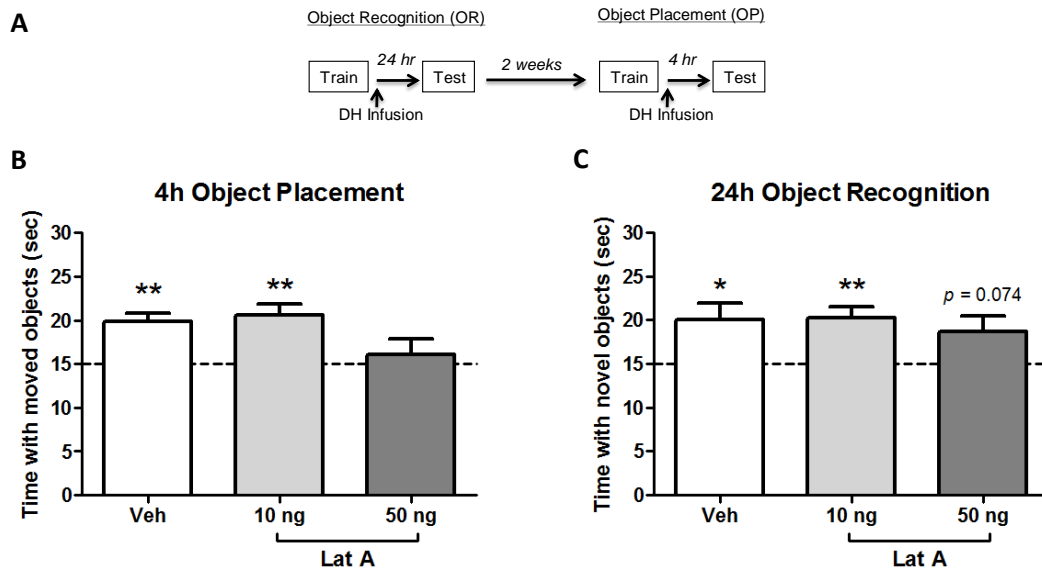
Figure 13. Actin polymerization inhibition alone impaired hippocampal memory consolidation. (A) Experimental design for behavioral tasks. The order of OR and OP testing were counterbalanced. (B) Mice receiving DH infusion of vehicle or 10 ng Latrunculin A showed a significant preference for the moved object 4 hour after OP training, suggesting no effect of this dose of Latrunculin A on spatial memory. However, 50 ng Latrunculin A impaired spatial memory. (C) Similarly, in OR, only 50 ng Latrunculin A impaired object memory consolidation, as vehicle- or 10 ng Latrunculin A-infused mice spent more time than chance with the novel object (*$p < 0.05$, **$p < 0.01$ relative to chance level of 15 seconds).

To test whether latrunculin A could block the memory-enhancing effects of G-1, we first needed to test the effects of latrunculin A alone on object recognition and spatial memory consolidation using the OR and OP tasks. We primarily needed to identify a dose of latrunculin A that had no effect on memory on its own so that any effect in combination with G-1 could be attributed to the interaction of the two compounds rather than a memory-impairing effect of latrunculin A. The latrunculin A doses, 100 ng/µl (50 ng/side) as a high concentration and 20

ng/µl (10 ng/side) as a low concentration, were selected based on previous studies demonstrating that intrahippocampal infusions of 500 ng/µl, but not 100 ng/µl, latrunculin A, impair object placement memory in rats (Nelson, et al., 2012) and basolateral amygdala infusions of 25 ng/µl latrunculin A block drug-associated memory (Young, et al., 2014).

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; Fig. 13B), suggesting intact spatial memory after treatment with a low dose of latrunculin A and impaired spatial memory after treatment with a high dose of latrunculin A. Similarly, 24 hours 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; Fig. 13C), indicating that intact object recognition memory after treatment with 10 ng, but not 50 ng, latrunculin A. However, one-way ANOVAs 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 potently impair memory consolidation. Given that mice infused with 50 ng/side 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/side latrunculin A has no effect on memory consolidation on its own, thereby allowing us to co-infuse this dose with G-1 in the next series of studies. Elapsed time to accumulate 30 seconds of exploration did not differ among the groups for either OP ($F_{(2,19)}$ = 3.179, p = 0.0644) or OR ($F_{(2,27)}$ = 0.6382, p = 0.5360).

Next, to determine a potential role for actin polymerization in GPER-mediated

hippocampal spine density regulation, ovxed mice received ICV infusion of vehicleor G-1 (8 ng) and DH infusion of vehicle or latrunculin A (10 ng/side) to form three groups: vehicle + vehicle, G-1 + vehicle, or G-1 + latrunculin A. Forty minutes later, mice were cervically dislocated and decapitated, and whole brains were collected for measurement of CA1 apical spine density. Analysis of Golgi-stained sections revealed a significant main effect of treatment on CA1 apical dendritic spine density ($F_{(2,26)} = 25.67$, $p = 0.0001$; Fig. 14A). ICV infusion of G-1 increased spine density relative to vehicle ($p < 0.001$) and DH infusion of latrunculin A blocked this effect (Fig. 14A), suggesting that G-1-induced spine density changes are dependent on actin polymerization.
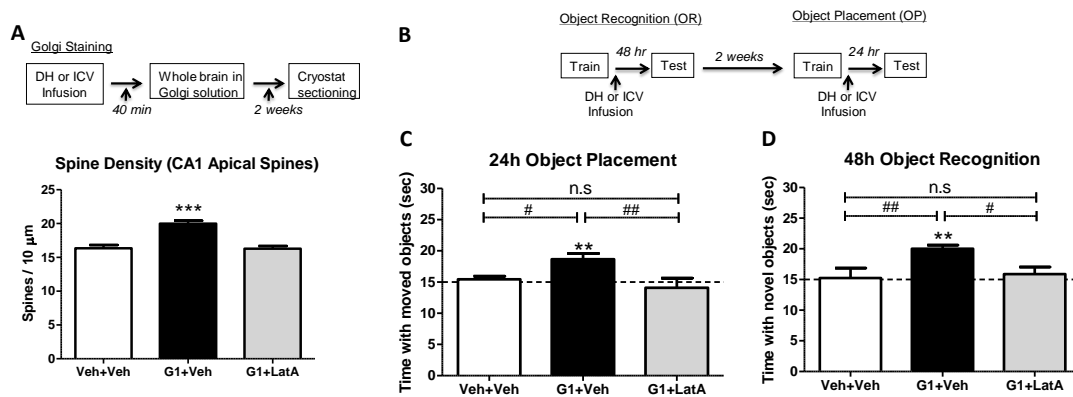
Figure 14. GPER-mediated memory enhancement and spine density alteration are dependent on hippocampal actin rearrangement. (A) ICV infusion of 8 ng G-1 significantly increased apical spine density, and DH infusion of the behaviorally-subeffective dose of 10 ng latrunculin A blocked this effect (***$p < 0.01$ relative to vehicle). (B) Experimental design for behavioral tasks. The order of OR and OP testing were counterbalanced. (C) Consistent with the spine data, ICV infusion of G-1 significantly enhanced spatial memory relative to vehicle and chance, and DH infusion of latrunculin A abolished this effect. (D) Similarly, latrunculin A prevented G-1 from enhancing object recognition memory relative to vehicle and chance (**$p < 0.01$ relative to chance; #$p < 0.05$, ##$p < 0.01$ relative to vehicle; n.s., Non-significant).

Next, we investigated a role for actin polymerization in GPER-mediated hippocampal memory enhancement. Ovxed mice received ICV and DH infusions, respectively, of vehicle + vehicle, 8 ng G-1 + vehicle, or G-1 + 10 ng/side latrunculin A. OR and OP retention was tested 48 and 24 hours later, respectively, as described. In both tasks, latrunculin A blocked the

memory-enhancing effects of G-1 (Fig. 14C, D). 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 + vehicle (moved object, $t_{(10)}$ = 0.9013, p = 0.3886; novel object, $t_{(10)}$ = 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 necessary 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 object (G-1 + vehicle, $p < 0.05$) and novel object (G-1 + vehicle, $p < 0.01$) than the vehicle + vehicle group, whereas the G-1 + latrunculin A group did not. Elapsed time to accumulate 30 seconds of exploration did not differ among the groups for either OP ($F_{(2,31)}$ = 0.8064, p = 0.4556) or OR ($F_{(2,27)}$ = 1.261, p = 0.2995). Together, these results suggest that GPER-mediated object recognition and spatial memory enhancement are dependent on actin polymerization in the hippocampus.

DISCUSSION

The goal of this dissertation is to provide insights into the cellular and molecular mechanisms through which GPER regulates hippocampal memory consolidation. We hypothesized that dendritic spines play a key role in GPER-mediated memory formation via cofilin- and JNK-mediated actin phosphorylation. The results suggest that actin rearrangement plays a pivotal role in GPER-mediated object recognition and spatial memory enhancement, as well as CA1 dendritic spine remodeling. Here, we used phosphorylation of the actin-binding protein cofilin as a proxy for actin regulation because cofilin is a key regulator of actin dynamics, and kinase-induced inactivation of cofilin via phosphorylation is necessary for spinogenesis and LTP maintenance (Babayan & Kramar, 2013; Chen, et al., 2007). The results suggest several novel findings about the effects of GPER activation and E_2 treatment in the DH of ovxed mice. First, GPER activation increases CA1 dendritic spine density and increases cofilin phosphorylation in a manner that depends on JNK activation. Second, E_2 increases cofilin phosphorylation in a manner that does not depend on GPER. Finally, the memory-enhancing and spinogenic effects of GPER activation are blocked by inhibition of actin polymerization. Collectively, these results provide the first evidence that GPER enhances hippocampal memory consolidation and regulates dendritic spine density in females by modulating actin dynamics via JNK-cofilin signaling (Fig. 15). The data also suggest that GPER does not function as an estrogen receptor to regulate actin polymerization. Together, this work supports the important role of GPER in mediating hippocampal morphology and memory consolidation, as well as the functional independence of GPER and E_2 within the DH. Because E_2 and GPER appear to employ different cell-signaling mechanisms to enhance memory consolidation (J. Kim, et al., 2016), these data may have important implications for the development of treatments that mimic

the beneficial cognitive effects of E2 without the dangerous side effects of current hormone

replacement therapies.
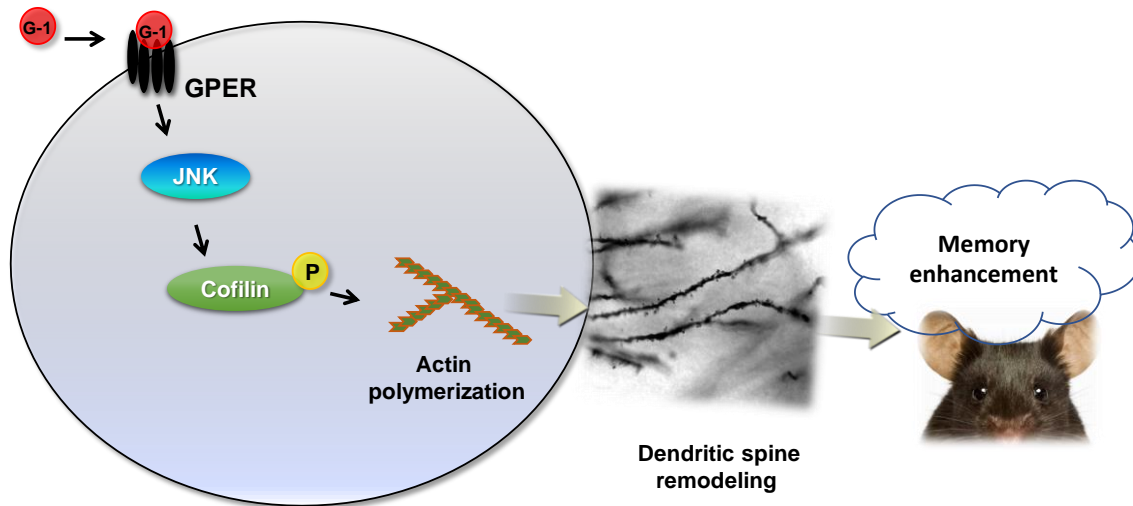
Figure 15. Proposed mechanisms involved in the role of actin polymerization in GPER-mediated hippocampal memory enhancement and spinogenesis in female mice. Hippocampal actin polymerization plays a necessary role in GPER-mediated hippocampal spine density alterations and memory consolidation. Also, GPER activation increases cofilin phosphorylation in a manner that depends on JNK activation.

Object Training Induced Dendritic Spine Remodeling in the Hippocampus

It is perhaps not surprising that certain stimuli, such as sensory input, stress, and

learning, influence synaptic connectivity and neuronal plasticity, given that dendritic spines are

considered a primary site of synaptic plasticity (Fiala, et al., 2002; Sala & Segal, 2014).

Numerous studies have attempted to link spines alterations with forms of physiological plasticity,

such as LTP and LTD, well-established synaptic models for memory formation and decline. For

example, LTP is associated with increased dendritic spine size and number, whereas LTD is

associated with decreased CA1 spine number (Matsuzaki, et al., 2004; Muller, et al., 2000; Zhou,

et al., 2004). Moreover, several studies have demonstrated that spine structural plasticity is

associated with learning experience. Fear conditioning and extinction, olfactory learning, and

spatial training affect spine formation and elimination in several brain regions, including layer-V pyramidal neurons in the mouse frontal association cortex, pyramidal neurons in the rat piriform cortex, and pyramidal neurons in rat CA1 (Knafo, et al., 2001; Lai, et al., 2012; Moser, et al., 1994). However, little is known about how object training affects dendritic spine density in the hippocampus. Therefore, we examined whether object training might increase CA1 pyramidal neuron spine density 40 minutes later. Although this time point is earlier than that used in other studies showing increased spine density 6 hours to 3 days after learning (Knafo, et al., 2001; Lai, et al., 2012; Moser, et al., 1994), we expected earlier spine remodeling here because a previous study reported that object training increased numerous elements of canonical Wnt/β-catenin signaling in mice within 5–30 minutes, including GSK3β, β-catenin, Cyclin D1, and Wnt 7a (Fortress, Schram, et al., 2013), which are involved in the regulation of dendritic spine formation (Ciani et al., 2011; Gogolla, Galimberti, Deguchi, & Caroni, 2009; Murase, Mosser, & Schuman, 2002). Forty minutes after training, apical CA1 dendritic spine density was significantly higher in object trained mice compared with home cage controls, suggesting that object training increased dendritic spinogenesis. Training increased dendritic spine density by 15% on apical pyramidal dendrites, similar to the increase previously observed in the piriform cortex following olfactory learning (Knafo, et al., 2001). Previous work suggests that learning-induced increases in synapse number are transient (Nimchinsky, et al., 2002); for instance, a twofold increase in hippocampal dentate spine density evident 6 hours after avoidance conditioning training had subsided by 72 hours (O'Malley, O'Connell, & Regan, 1998). Because the present study examined just a single time point, the transience of the object learning-induced increase in CA1 spine density is unknown. Thus, further studies will be necessary to examine the temporal dynamics of CA1 dendritic spine remodeling in response to object learning. In addition, spine

morphology alteration analysis associated with learning could provide better understanding of spine dynamics. Modulation of an epigenetic factor, BAF53b overexpression, promotes the formation of new dendritic spines in the lateral amygdala, particularly of the thin subtype, after fear learning (Yoo et al., 2017). Thin-type spines represent synapses with lower synaptic strength with a low AMPAR/NMDAR ratio compared to mushroom-type spines with a higher AMPAR/NMDAR ratio (Das et al., 2008; Harris & Stevens, 1989; Yasumatsu, Matsuzaki, Miyazaki, Noguchi, & Kasai, 2008). Thin-type spines can be converted by LTP and learning to mushroom-type spines (Holtmaat et al., 2005; Kasai, Fukuda, Watanabe, Hayashi-Takagi, & Noguchi, 2010), suggesting highly thin subtype spines are highly plastic. Therefore, further studies of spine subtype analysis could provide better comprehension about new spine outgrowth after object learning in the hippocampus.

The Role of GPER Activation and JNK signaling in Hippocampal Dendritic Spine Density and Cofilin Phosphorylation

Previously, our laboratory showed that G-1 mimicked the beneficial effects of E_2 on object recognition and spatial memory consolidation (J. Kim, et al., 2016) and reported that DH infusion of E_2 increases CA1 dendritic spine density in the DH (Tuscher, et al., 2016). Thus, here we sought to determine if GPER activation might enhance memory consolidation by increasing CA1 dendritic spine density. DH G-1 infusion increased CA1 apical dendritic spine density within 40 minutes, which is consistent with a previous report of increased CA1 dendritic spine density 40 minutes after systemic G-1 treatment (Gabor, et al., 2015). The timing of these effects are consistent with those of E_2, as systemic injection or DH E_2 infusion increases DH CA1 dendritic spine density 30 minutes and 2 hours later (Inagaki, et al., 2012; MacLusky, Luine,

Hajszan, & Leranth, 2005; Tuscher, et al., 2016). Together, these data indicate that both E_2 and GPER activation rapidly regulate hippocampal dendritic spine density.

Because rapid activation of cell-signaling kinases in the DH is necessary for E_2 to increase CA1 dendritic spine density, we also examined the involvement of cell signaling mechanisms in G-1-induced spinogenesis. We first determined the extent to which GPER activation regulates cofilin phosphorylation in the DH. Cofilin is an important regulator of actin dynamics, and E_2 phosphorylates cofilin by activating the small GTPase RhoA (Kramar, et al., 2009). Here, we found a transient increase in cofilin phosphorylation 5 and 15 minutes after DH G-1 infusion that returned to baseline 30 minutes after infusion. These data indicate that G-1 rapidly and transiently phosphorylates cofilin in the DH. Because a few studies in breast cancer cell lines reported that G-1 can act in a GPER-independent manner (Kang et al., 2010; C. Wang, Lv, Jiang, & Davis, 2012), we used G-15, a selective GPER antagonist to confirm that G-1-induced cofilin phosphorylation and spinogenesis occur via GPER activation. Consistent with actions via GPER, G-15 blocked G-1's effects on cofilin phosphorylation and CA1 spine density, suggesting that G-1-induced phosphorylation of cofilin and spine remodeling occur via GPER activation.

Because we previously reported that G-1 led to rapid phosphorylation of both isoforms of the MAP kinase JNK in the DH (J. 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 is involved in the regulation of 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 hippocampal CA1 pyramidal neurons (Matsuzaki et al., 2001), JNK signaling likely

plays an important role in synaptic transmission. Therefore, we expected JNK to be involved in GPER's effects on spine morphology and hippocampal memory consolidation. 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 (J. Kim, et al., 2016). Here, we used the same dose of SP600125 to show that JNK inhibition abolished G-1's effects on cofilin phosphorylation and CA1 spine density. These data indicate that JNK signaling is necessary for G-1-induced cofilin phosphorylation and CA1 dendritic spinogenesis. Together with our previous report (J. Kim, et al., 2016), these findings suggest an essential role for JNK-mediated spine remodeling in the memory-enhancing effects of GPER.

The Role of GPER Activation in E_2-induced Cofilin Phosphorylation

GPER is a former orphan G-protein-coupled receptor previously named GPR-30. It was designated an estrogen receptor after demonstration that E_2 and other estrogenic compounds bound the receptor with a high affinity in various human cell lines (Funakoshi, et al., 2006; Thomas & Dong, 2006). However, not all investigators believe GPER to be a true estrogen receptor, but rather a collaborator in mediating the biological actions of estrogens (Levin, 2009; Langer et al., 2010). Consistent with the idea that GPER does not function as a canonical estrogen receptor, we recently showed that GPER and E_2 do not enhance memory via the same cell signaling mechanisms. E_2 and agonists of ERα and ERβ require ERK activation in the DH to enhance object and spatial memory consolidation in ovxed female mice (M. I. Boulware, et al., 2013; Fernandez, et al., 2008; Fortress, Fan, et al., 2013), however, our data indicate that activation of JNK, not ERK, is essential for the memory-enhancing effects of GPER (J. Kim, et al., 2016). Moreover, G-15 does not abolish the memory-enhancing effects of E_2 in OR or OP

among ovxed mice (J. Kim, et al., 2016), suggesting that GPER activation is not necessary for E_2 to enhance hippocampal memory consolidation. However, it remains possible that GPER mediates other effects of E_2 on hippocampal function. Therefore, we explored a possible role for GPER activation in E_2-mediated cofilin phosphorylation. Consistent with our previous study, G-15 infusion did not block E_2-mediated cofilin phosphorylation in the DH, indicating that GPER activation is not necessary for E_2 to regulate cofilin activation. This finding is supported by other studies showing that E_2 and GPER may affect spines independently. For example, one study found that ERα and ERβ are responsible for cofilin-related actin polymerization, whereas GPER instead interacts with TrkB receptors to stimulate Akt/mTOR-mediated protein synthesis (Briz & Baudry, 2014). In addition, although E_2 reportedly binds GPER with high affinity (Funakoshi, et al., 2006; Moriarty, et al., 2006; Prossnitz, et al., 2007b; Revankar, et al., 2005), many studies have found that GPER acts independently of E_2 in several *in vitro* cell systems, including COS-7 cells, CHO (Chinese hamster ovary) cells, rat aortic vascular endothelial cells, and breast cancer cells (Ding, Hussain, Chorazyczewski, Gros, & Feldman, 2014; Madak-Erdogan et al., 2008; Otto et al., 2008; Pedram, Razandi, & Levin, 2006).

Interestingly, the level of E_2-induced cofilin phosphorylation observed here was relatively smaller and more transient than that mediated by GPER. G-1 increased DH cofilin phosphorylation relative to vehicle both 5 and 15 minutes after DH infusion, however, the E_2-induced increase lasted only 5 minutes. In addition, G-1 increased the levels of DH cofilin phosphorylation by about 60-70% relative to vehicle, whereas the E_2-induced increase was only about 30%. Even considering that G-1 mobilizes intracellular calcium ($t_{1/2} \approx 30$ s) slightly slower than E_2 ($t_{1/2} \approx 2$ s) (Bologa, et al., 2006), the GPER-induced increase in cofilin phosphorylation was almost double the size of that produced by E_2. If E_2 activates cofilin by binding to GPER,

then it should have produced levels of cofilin phosphorylation at least comparable to G-1 infusion. Therefore, these results also support the conclusion that E_2 and GPER independently regulate cofilin-mediated actin polymerization in the DH as a mechanism to regulate dendritic spine density.

The Role of Actin Polymerization in GPER-mediated Spine Density Alterations and Memory Enhancement in the Hippocampus

Finally, to tie the effects of GPER activation on cofilin and dendritic spine density with its beneficial effects on hippocampal memory consolidation, we sought to determine whether inhibiting actin polymerization could prevent GPER from enhancing memory. Latrunculin A was used to inhibit actin polymerization because this compound binds to G-actin and prevents actin polymerization, which decreases spine number (Penzes & Cahill, 2012; Yarmola, et al., 2000). Moreover, intrahippocampal infusion of latrunculin A blocks object placement memory in rats (Nelson, et al., 2012), suggesting that actin polymerization is essential for object memory formation. We first sought to establish a dose of latrunculin A that had no detrimental effects on memory consolidation on its own to ensure that any effects seen in combination with GPER agonists result from an interaction between the inhibitor and hormone compound, rather than a more general memory impairment induced by the inhibitor. DH infusion of 50 ng/side blocked OP memory consolidation relative to chance, and tended to block OR memory consolidation, which is consistent with previous studies in which latrunculin A impaired memory after infusion into the hippocampus or basolateral amygdala (Nelson, et al., 2012; Young, et al., 2014) and other studies focusing on the functional roles of actin polymerization in postsynaptic AMPA receptor trafficking, object placement memory, and drug-associated memory (Li, et al., 2015;

Nelson, et al., 2012; Young, et al., 2014; Zhou, et al., 2001). Importantly, 10 ng latrunculin A did not impair memory in either task, providing us with a dose to use in combination with G-1. DH infusion of 10 ng/side latrunculin A prevented G-1 from increasing CA1 dendritic spine density and blocked its memory-enhancing effects, suggesting that actin rearrangement is necessary for G-1-induced spine density changes and hippocampal memory enhancement. These data provide the first evidence that actin rearrangement is necessary for GPER-mediated hippocampal memory. 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, we believe these data provide promising new avenues for the development of novel therapies that mimic the memory-enhancing effects of estrogens without harmful side effects.

Further Studies and Conclusions

The experiments of this dissertation extend our previous findings that GPER activation enhances hippocampal memory via the JNK signaling pathway by showing a key involvement of JNK in mediating GPER's effects on cofilin phosphorylation and dendritic spine density. Given how little is known about the role of JNK in cofilin signaling, it is interesting that JNK inhibition abolished GPER activation effects on cofilin phosphorylation. In the nervous system, JNK plays a pivotal role in synaptic plasticity, neuronal regeneration, and brain development (Tararuk et al., 2006; Waetzig, Zhao, & Herdegen, 2006). Evidence also shows that short-term JNK activation facilitates hippocampal memory and synaptic plasticity, whereas prolonged JNK activation leads to memory deficits and neurodegeneration (Sherrin, Blank, & Todorovic, 2011). However, conflicting results suggest that the role of hippocampal JNK in short-term learning and memory

is not entirely clear (Bevilaqua, Kerr, Medina, Izquierdo, & Cammarota, 2003). JNK has more than 60 substrates, including a variety of nuclear transcription factors such as c-Jun, ATF2, and Elk-1, as well as cytoplasmic substrates such as cytoskeletal proteins and mitochondrial proteins like Bcl-2 and Bcl-xl (Antoniou & Borsello, 2012). However, much less is known about the role of JNK in mediating the cofilin signaling pathway. Therefore, the JNK-cofilin signaling relationship is worthy of further study. In vascular endothelial cells, JNK inhibition decreased phospho-cofilin levels, suggesting that JNK in some way enhances cofilin phosphorylation (Slee & Lowe-Krentz, 2013). In addition, in cultures of hippocampal neurons, JNK activation at axon tips facilitates axon elongation by increasing cofilin phosphorylation and promoting actin polymerization (T. Sun et al., 2013). In contrast, other data indicate that platelet-derived growth factor-BB-induced dephosphorylation of cofilin can be promoted by JNK in rat aortic smooth muscle cells (Won et al., 2008). Therefore, the role of JNK signaling in cofilin signaling is still unclear and may be tissue-specific. Given how little is known about the role of JNK in hippocampal cofilin signaling and related memory consolidation, one possible future direction would be to more thoroughly elucidate the molecular mechanisms through which JNK-mediated cofilin signaling regulates dendritic spine remodeling and hippocampal memory.

The results of this dissertation also support the independence of GPER and E_2 within the DH in mediating hippocampal spine morphology and memory consolidation. Therefore, future studies could further probe this relationship. Based on our current and previous results (M. I. Boulware, et al., 2013; J. Kim, et al., 2016), we conclude E_2 regulates hippocampal synaptic plasticity and memory consolidation via ERα and ERβ, not GPER. Our current and previous data indicate that GPER regulates hippocampal plasticity and memory on its own (J. Kim, et al., 2016), which suggests that GPER does not function as an estrogen receptor in the dorsal

hippocampus. If not E_2, then a possible alternative natural ligand for GPER might be aldosterone. One report indicated a potential role of aldosterone in GPER activation in vascular smooth muscle cells (G. C. Brailoiu et al., 2013; Gros, Ding, Liu, Chorazyczewski, & Feldman, 2013), although this claim requires further investigation (Filardo & Thomas, 2012). Interestingly, several studies indicate that GPER acts independently of E_2 (Ding, et al., 2014; Otto, et al., 2008; Pedram, et al., 2006). However, many other studies show that E_2 activates GPER (Langer et al., 2010; Moriarty, et al., 2006; Prossnitz, Arterburn, & Sklar, 2007a; Revankar, et al., 2005; Thomas, et al., 2005). Therefore, an alternative hypothesis is that the activation of intracellular ERs may inhibit GPER activation. Such inhibition may arise because E_2 has a higher binding affinity for ERα and ERβ than for GPER, as demonstrated by competitive radiometric binding assays showing much lower K_d values for E_2 on ERα (0.30 nM) and ERβ (0.90 nM) in human endometrial cancer (HEC-1) cells than for E_2 on GPER (3.0 nM) in human embryonic kidney (HEK) 293 cells (J. Sun et al., 1999; Thomas, et al., 2005). In addition, GPER1 and the classical ERs may act in a parallel manner, such that GPER1 or intracellular ERs might signal to the same output via an overlapping subset of signals (Hadjimarkou & Vasudevan, 2018). Undoubtedly, additional studies will be necessary to elucidate how E_2 binding at the intracellular ERs and GPER acts independently and/or in conjunction to regulate hippocampal function.

In conclusion, the studies of this dissertation provide a better understanding of the cell-signaling mechanisms through which the membrane ER called GPER regulates hippocampal dendritic spine density and memory consolidation. This work also provides another test of the notion that GPER acts independently from ERα or ERβ, even though it mimics the beneficial effects of 17β-estradiol on spine remodeling and hippocampal memory consolidation in ovariectomized female mice. Therefore, these studies can provide valuable insights into the role

of GPER in mediating hippocampal morphology and memory consolidation, and may suggest first steps towards new therapeutics that more safely and effectively reduce memory decline in menopausal women. The massive loss of estrogens at menopause significantly increases the risk of memory deficiency and Alzheimer's disease (AD) in women (Yaffe et al., 2007; Zandi et al., 2002); accordingly 3.4 million out of the 5.2 million AD patients are women (Alzheimer's Association, 2012). The risk of menopause-related memory decline and AD in women creates enormous problems not only for the individual who is suffering, but also for the health care system, families, and the federal budget. In 2012, the costs of patient care for AD and other dementias was estimated at $200 billion and are predicted to rise to $1.1 trillion by 2050 (Alzheimer's Association, 2012).

Although estrogen replacement can reduce the risk of menopause-related memory decline and AD in women, current hormone therapies are not recommended because of detrimental side effects such as increased risk of cancer, heart disease, and stroke (Coker et al., 2010; Rossouw et al., 2002). These side effects are thought to result from interactions between E_2 and the classical ERs ERα and ERβ. ERα and ERβ activation are involved in certain types of cancer (Burns & Korach, 2012; Deroo & Korach, 2006), for example, nuclear ERα and ERβ expression increased or stayed constant during breast cancer progression (Filardo et al., 2006). In contrast, GPER expression is decreased in cancer cell lines, where it acts independently from ERα and ERβ (Filardo & Thomas, 2012). GPER activation has also been shown to suppress cell proliferation in ovarian cancer cell lines (Ignatov et al., 2013). Given that GPER activation mimics the beneficial effects of E_2 memory, but appears to exert its effects independently from E_2 by triggering different cell-signaling mechanisms, this receptor could be a potential target for the development of new hormone replacement therapies that exhibit the memory-enhancing

effects of intracellular ER activation without cancerous side effects. Therefore, a better

understanding the mechanisms through which GPER regulates memory may help to produce

safer and more effective treatments for reducing memory decline in menopausal women.

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recognition. *J Neurosci, 32*(7), 2344-2351.

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EDUCATION

University of Wisconsin-Milwaukee, WI, USA   Sep. 2011 - present
Department of Psychology
Ph.D. program in Experimental Psychology

University of Wisconsin-Milwaukee, WI, USA   Sep. 2011 – Dec. 2014
Department of Psychology
Major in Psychology, M.S.

Seoul National University, Seoul, Korea   Sep. 2009 - Aug. 2011
Department of Agricultural Biotechnology
Major in Biomodulation, M.S.

Yonsei University, Seoul, Korea   Mar. 2001 - Aug. 2008
Department of Biochemistry
Major in Biochemistry, B.S.

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RESEARCH AND LABORATORY EXPERIENCE

University of Wisconsin-Milwaukee   Sep. 2011 - present
Department of Psychology
Supervisor: Prof. Karyn Frick
Ph.D. Graduate student
- *Project*: Identifying the molecular mechanisms through which estrogens regulate memory formation, with a particular focus on the role of GPER in mediating the effects of 17β-estradiol on memory consolidation.
- *Methods*: Intracranial cannula implantation and drug infusion, stereotaxic surgery, ovariectomy, object recognition, object placement, Western blotting, qPCR, golgi staining, spine counting quantification(Neurolucida), Accell siRNA

Seoul National University   Sep. 2009 - Aug.2011
Department of Agricultural Biotechnology
Supervisor: Prof. Hyong Joo Lee
Master's Graduate student
- *Project*: Identifying the effect of dehydroglyasperin C derived from licorice on expression of MAPK phosphatase 1and inflammation-mediated neurodegeneration / Identifying the role of Nrf2 in the inflammation-mediated reactive microgliosis
- *Methods*: primary cortical neuron culture derived from mouse embryo, BV-2 murine microglia culture, cell viability assay, DNA/RNA transfection, EMSA, transcriptional activity assay using luciferase reporter gene, RT-PCR, Western blot, ELISA, In vitro

kinase activity assay, immunoflouresence staining, transgenic mouse genotyping (3XTg AD, Nrf2 KO, Keap1 KO, TRPV1 KO), oral gavage, I.P. injection

<u>University of Alberta</u>   Jan. 2007 - May 2007
Department of Biochemistry
Supervisor: Prof. Richard Fahlman
Independent Research Project - BIOCH 498 honors student
  - *Project*: Enzymatic transfer of an unnatural amino acid by leucyl/phenylalanyl-tRNA-protein transferase
  - *Methods*: mass Spectroscopy Assay for L/F-Transferase activity, Wizard® Plus Midipreps DNA Purification System, Ni-NTA purification system, QuikChange® Site-Directed Mutagenesis Kit

<u>Yonsei University</u>   Oct. 2002 - Apr. 2003
Department of Biochemistry
Biomolecular NMR Laboratory
Supervisor: Prof. Weontae Lee
Research assistant
  - *Project*: Analysis of the Cyclic-MSH-11 3D structure, one of the agonists of the Melanocortin receptor
  - *Methods*: producing NMR samples(cell culture, harvest, and lysis), His-Tag Proteins/Nickel column purification, chromatography, SDS-PAGE

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PRESENTATIONS

<u>University of Wisconsin - Milwaukee</u>   Apr. 2018
Association of Graduate Students in Psychology Symposium
  - The role of actin polymerization in GPER-mediated hippocampal memory enhancement in female mice

<u>University of Wisconsin - Milwaukee</u>   Jan. 2015
Seminar in Neuroscience
  - Distinct Effects of Estrogen Receptor Inhibition on Hippocampal Memory in Female Mice

<u>University of Wisconsin - Milwaukee</u>   May 2014
Seminar in Neuroscience
  - Role of G-protein-coupled Estrogen Receptor (GPER) on Hippocampal Memory and Cell Signaling Cascades in Female Mice

<u>University of Wisconsin - Milwaukee</u>   Apr. 2014
Association of Graduate Students in Psychology Symposium
  - G-protein-coupled Estrogen Receptor (GPER/GPR30): The Estrogen Receptor That Doesn't Act Like an Estrogen Receptor

<u>University of Wisconsin - Milwaukee</u>   Apr. 2013
Association of Graduate Students in Psychology Symposium

- Role of G-protein-coupled Estrogen Receptor (GPER) on Hippocampal Memory and Cell Signaling Cascades in Female Mice

<u>Seoul National University</u>   May 2011
Biomodulation Spring Thesis Seminar Presentation
- Dehydroglyasperin C Derived from Licorice Increases the Expression of MAP Kinase Phosphatase 1 and Suppresses Inflammation-Mediated Neurodegeneration

<u>University of Alberta</u>   May 2007
Undergraduate Research Day Presentation
- Enzymatic transfer of an unnatural amino acid by leucyl/phenylalanyl-tRNA-protein transferase.

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POSTERS

<u>Organization for the Studay of Sex Differences</u>   Apr. 2018
Annual meeting
- **Kim, J.**, Schalk, J.C., Koss, W.A., Frick, K.M. (2018). The role of actin polymerization in GPER-mediated hippocampal memory enhancement in female mice. *Society for Neuroscience Abstracts*, Poster 159.04.

<u>Society for Neuroscience</u>   Nov. 2017
Annual meeting, Neuroscience 2017
- **Kim, J.**, Schalk, J.C., Koss, W.A., Frick, K.M. (2017). The role of actin polymerization in GPER-mediated hippocampal memory enhancement in female mice. *Society for Neuroscience Abstracts*, Poster 159.04.

<u>Society for Neuroscience</u>   Nov. 2016
Annual meeting, Neuroscience 2016
- **Kim, J.**, Julia, S.S., Boulware, M.I., Frick, K.M. (2016). Effects of agonism and silencing
- of G-protein-Coupled Estrogen Receptor (GPER) on hippocampal memory and underlying cell-signaling mechanisms in female mice. *Society for Neuroscience Abstracts*, Poster 719.11.

<u>University of Wisconsin-Miwaukee</u>   Mar. 2016
Neuroscience Mini-symposium
- **Kim, J.**, Fortress, A.M., Frick, K.M. (2015). The G-protein-coupled estrogen receptor (GPER/GPR30) modulates cellsignaling proteins that regulate actin polymerization in the dorsal hippocampus of female mice.

<u>Society for Neuroscience</u>   Oct. 2015
Annual meeting, Neuroscience 2015
- **Kim, J.**, Fortress, A.M., Frick, K.M. (2015). The G-protein-coupled estrogen receptor (GPER/GPR30) modulates cellsignaling proteins that regulate actin polymerization in the dorsal hippocampus of female mice. *Society for Neuroscience Abstracts*, Poster 614.11.

<u>University of Wisconsin-Miwaukee</u>   Oct. 2015
Annual pre- SfN minisymposium

- **Kim, J.**, Fortress, A.M., Frick, K.M. (2015). The G-protein-coupled estrogen receptor (GPER/GPR30) modulates cellsignaling proteins that regulate actin polymerization in the dorsal hippocampus of female mice.

Society for Neuroscience   Nov. 2014
Annual meeting, Neuroscience 2014
- **Kim, J.**, Julia, S.S., Frick, K.M. (2014). Distinct effects of estrogen receptor inhibition on novel object recognition and spatial memory consolidation in ovariectomized mice. *Society for Neuroscience Abstracts*, Poster 451.11.

University of Wisconsin-Miwaukee   Nov. 2014
Annual pre- SfN minisymposium
- **Kim, J.**, Julia, S.S., Frick, K.M. (2014). Distinct effects of estrogen receptor inhibition on novel object recognition and spatial memory consolidation in ovariectomized mice.

Medical College of Wisconsin   Apr. 2014
Milwaukee SfN Meeting
- **Kim, J.**, Boulware, M.I., Frick, K.M. (2014). Role of G-protein-coupled estrogen receptor (GPER/GPR30) in hippocampal memory and cell signaling in female mice.

Society for Neuroscience   Nov. 2013
Annual meeting, Neuroscience 2013
- **Kim, J.**, Boulware, M.I., Frick, K.M. (2013). Role of G-protein-coupled estrogen receptor (GPER/GPR30) in hippocampal memory and cell signaling in female mice. *Society for Neuroscience Abstracts*, Poster 376.05.

University of Wisconsin-Miwaukee   Nov. 2013
Annual pre- SfN minisymposium
- **Kim, J.**, Boulware, M.I., Frick, K.M. (2013). Role of G-protein-coupled estrogen receptor (GPER/GPR30) in hippocampal memory and cell signaling in female mice.

Seoul National University   Mar. 2011
2nd Biomodulation Poster Retreat, Biomodulation International Symposium
- **Kim, J**., Kim, J., Shim, J., Lee, K.W., Lee, H.J. (2011) Dehydroglyasperin C derived from licorice increases the expression of MAP kinase phosphatase 1 and suppresses inflammation-mediated neurodegeneration.

Seoul National University   Mar. 2011
2nd Biomodulation Poster Retreat, Biomodulation International Symposium
- Kim, H.W., Kim, J., **Kim, J.**, Lee, S., Choi, B.R., Han, J.S., Lee, K.W., Lee, H.J. (2011) 3,3'-Diindolylmethane inhibits lipopolysaccharide-induced microglial hyperactivation and attenuates brain inflammation.

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PATENT

A composition comprising dehydroglyasperin C for the treatment of the decline or damage of cognitive function. **Kim, J.K.**, Kim, J.Y., Lim, S.S., Lee, K.W., Lee, H.J. Korean Patent 1017119700000 (2017)
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TEACHING EXPERIENCE

     University of Wisconsin - Milwaukee   Spring semester 2014
     Psych 101: Introduction to Psychology
       -    Guest lecture: Stress and Health

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HONORS / AWARDS

     Organization for the Study of Sex Difference   Apr. 2018
     OSSD 2018, NIH-Sponsored Travel Award

     University of Wisconsin - Milwaukee   Nov. 2017
     Graduate School Graduate Student Travel Awards

     University of Wisconsin - Milwaukee   2016-2017
     Distinguished Dissertation Fellowship (DDF)

     University of Wisconsin - Milwaukee   Oct. 2015
     Graduate School Graduate Student Travel Awards

     University of Wisconsin - Milwaukee   2014-2015
     Distinguished Graduate Student Fellowship (DGSF)

     University of Wisconsin - Milwaukee   Jun.-Aug. 2014
     Department of Psychology Summer Graduate Research Fellowship

     University of Wisconsin - Milwaukee   Nov. 2013
     Graduate School Graduate Student Travel Awards

     Seoul National University   2010-2011
     Graduate Scholarship

     Seoul National University   2009
     Brain Korea 21 Scholarship

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PROFESSIONAL AFFILIATIONS

     Society for Neuroscience   - Student member   Sep. 2011 - present

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ASSISTANTSHIPS

     Research Assistantship   Sep. 2017 – May 2018
     Distinguished Dissertation Fellowship   Sep. 2016 - May 2017
     Teaching Assistantship   Sep. 2015 - May 2016
     Distinguished Graduate Student Fellowship   Sep. 2014 - May 2015
     Teaching Assistantship   Sep. 2013 - Aug. 2014

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PUBLICATIONS

Hanson, A.M., Perera, I.S., **Kim, J.**, Pandey, R.K., Sweeney, N., Lua, X., Imhoff, A., Mackinnon, A.C., Frick, K.M., Donaldson, W.A., Sem, D.S. (2018) A-C estrogens as potent and selective estrogen receptor-beta agonists (SERBAs) to enhance memory consolidation under low-estrogen conditions. *J Med Chem*, 61(11):4720-4738.

Frick, K.M., **Kim, J.** (2018) Mechanisms underlying the rapid effects of estradiol and progesterone on hippocampal memory consolidation in female rodents. *Horm Behav*, pii: S0018-506X(18)30088-6.

Frick, K.M., **Kim, J.,** Koss, W.A. (2018) Estradiol and hippocampal memory in female and male rodents. *Curr Opin Behav Sci*, 23:65-74.

Frick, K.M., Tuscher, J.J., Koss, W.A., **Kim, J.**, Taxier, L.R. (2018) Estrogenic regulation of memory consolidation: A look beyond the hippocampus, ovaries, and females. *Physiol Behav,* 187:57-66.

Kim, J., Frick, K.M. (2017) Distinct effects of estrogen receptor antagonism on object recognition and spatial memory consolidation in ovariectomized mice. *Psychoneuroendocrinology*, 85:110-114.

Kim, J., Szinte, J.S., Boulware, M.I., Frick, K.M. (2016) 17β-Estradiol and agonism of G-protein-coupled estrogen receptor enhance hippocampal memory via different cell-signaling mechanisms. *J Neuro,* 36(11):3309-21.

Frick, K.M., **Kim, J.**, Tuscher, J.J., Fortress, A.M. (2015) Sex steroid hormones matter for learning and memory: estrogenic regulation of hippocampal function in male and female rodents. *Learn Mem,* 22(9):472-93.

Tuscher, J.J., Fortress, A.M., **Kim, J.**, Frick, K.M. (2015) Regulation of object recognition and object placement by ovarian sex steroid hormones. *Behav Brain Res,* 285:140-57.

Fortress, A.M., **Kim, J**., Poole, R.L., Gould, T.J., Frick, K.M. (2014) 17β-estradiol regulates histone alterations associated with memory consolidation and increases *Bdnf* promoter acetylation in middle-aged female mice. *Learn Mem,* 21(9):457-67.

Kim, H.W., Kim, J., **Kim, J.**, Lee, S., Choi, B.R., Han, J.S., Lee, K.W., Lee, H.J. (2014). 3,3'-Diindolylmethane inhibits lipopolysaccharide-induced microglial hyperactivation and attenuates brain inflammation. *Toxicol Sci,* 137(1):158-67.

Kim, J., **Kim, J.**, Shim, J., Lee, C.Y., Lee, K.W., Lee, H.J. (2014). Cocoa phytochemicals: recent advances in molecular mechanisms on health. *Crit Rev Food Sci Nutr*, 54(11):1458-72.

Kim, J., Kim, J., Shim, J., Lee, S., Kim, J., Lim, S.S., Lee, K.W., Lee, H.J. (2013). Licorice-derived dehydroglyasperin C increases MKP-1 expression and suppresses inflammation-mediated neurodegeneration. *Neurochem Int,* 63(8), 732-740.

Kim, J., Lee, S., Shim, J., Kim, H. W., **Kim, J.**, Jang, Y. J., Yang, H., Park, J., Choi, S. H., Yoon, J. H., Lee, K. W., & Lee, H. J. (2012). Caffeinated coffee, decaffeinated coffee, and the phenolic phytochemical chlorogenic acid up-regulate NQO1 expression and prevent H_2O_2-induced apoptosis in primary cortical neurons. *Neurochem Int,* 60(5), 466-474.

Jang, Y. J., Kim, J., Shim, J., **Kim, J.**, Byun, S., Oak, M. H., Lee, K. W., & Lee, H. J. (2011). Kaempferol attenuates 4-hydroxynonenal-induced apoptosis in PC12 cells by directly inhibiting NADPH oxidase. *J Pharmacol Exp Ther*, 337(3), 747-754.

Koh, K.[1], **Kim, J.**[1], Jang, Y. J., Yoon, K., Cha, Y., & Lee, H. J. (2011). Transcription factor Nrf2 suppresses LPS-induced hyperactivation of BV-2 microglial cells. *J Neuroimmunol*, 233(1-2), 160-167.
[1]:co-first author.
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REFERENCES

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