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The Role of G-protein Coupled Estrogen Receptor (GPER/GPR30) in Hippocampal Memory and Cell Signaling in Female Mice

Jae Kyoon Kim
University of Wisconsin-Milwaukee

THE ROLE OF G-PROTEIN COUPLED ESTROGEN RECEPTOR (GPER/GPR30) IN HIPPOCAMPAL MEMORY AND CELL SIGNALING IN FEMALE MICE

by

Jae Kyoon Kim

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ABSTRACT

THE ROLE OF G-PROTEIN COUPLED ESTROGEN RECEPTOR (GPER/GPR30) IN HIPPOCAMPAL MEMORY AND CELL SIGNALING IN FEMALE MICE

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The loss of estrogens at menopause significantly increases a woman's risk of memory loss and Alzheimer's disease because estrogens are essential trophic factors for the hippocampus. However, current hormone replacement therapies are not recommended to reduce age-related memory decline because of their adverse side effects. To develop better hormone replacement therapies, it is essential to understand the mechanisms through which estrogens regulate memory. Our laboratory has demonstrated that the ability of 17β-estradiol (E_2) to enhance hippocampal memory depends on the rapid activation of extracellular-signal-regulated kinase (ERK), which occurs through activation of ERα and ERβ. The G-protein coupled estrogen receptor (GPER) is a novel membrane estrogen receptor, expressed in areas of the brain important for learning and memory such as the hippocampus. However, little is known about the role of dorsal hippocampal (DH) GPER in hippocampal memory consolidation and cell signaling. Here, the present study tested the roles of GPER in regulating hippocampal memory consolidation and cell signaling in young female mice. DH infusion of the GPER agonist, G-1, enhanced object recognition and spatial memory consolidation in ovariectomized female mice. DH infusion of the GPER antagonist, G-15, blocked the memory-enhancing effects of G-1, suggesting that

GPER activation mimics the beneficial effects of E_2 on hippocampal memory. Interestingly, however, G-1 did not increase ERK phosphorylation like E_2, but instead significantly increased phosphorylation of the c-Jun N-terminal kinase (JNK) in the DH, suggesting that the molecular mechanisms underlying the memory-enhancing effects of GPER activation may differ from those of ERα and ERβ activation. Consistent with this notion, DH infusion of the JNK inhibitor, SP600125, blocked G-1-induced memory enhancement and JNK phosphorylation, whereas the ERK inhibitor, U0126, did not. Finally, we showed that DH infusion of SP600125 or G-15 did not prevent E_2 from enhancing memory and activating ERK, demonstrating that the memory-enhancing effects of E_2 are not dependent on JNK or GPER activation in the DH. These results indicate that GPER regulates memory independently from ERα and ERβ by activating JNK signaling, rather than ERK signaling. Together, the data suggest that GPER does not function as an estrogen receptor in the DH. As such, this study identifies GPER as a putative new target for reducing memory decline in menopausal women without the detrimental side effects of currently available treatment options

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Introduction

The massive loss of estrogens at menopause significantly increases the risk of memory deficiency and Alzheimer's disease (AD) in women (Zandi et al., 2002; Yaffe et al., 2007). Out of the 5.2 million Alzheimer's disease patients, 3.4 million are women (Alzheimer's Association, 2012), and this number will continue to increase due to the aging of the baby boomer generation. In 2012, the costs of patient care for AD and other dementias is estimated at $200 billion and are projected to rise to $1.1 trillion by 2050 (Alzheimer's Association, 2012). Estrogen therapies can decrease the risk of menopause-related memory decline and AD in women (Yaffe et al., 1998; Zandi et al., 2002), however these treatments are accompanied by increased risk of breast cancer, heart disease, and stroke (Rossouw et al., 2002). The effects of estrogens in the hippocampus are important to study because hippocampus dysfunction leads to memory loss (deToledo-Morrell et al., 1988). Unfortunately, the mechanisms underlying the beneficial effects of estrogens on memory are not fully understood. These beneficial effects may be mediated by intracellular estrogen receptors (ERα and ERβ) or membrane-bound ERs (e.g., G-protein coupled estrogen receptor; GPER) (Waters et al., 2011). Although some evidence suggests an important role of intracellular ERs (ERα and ERβ) in memory formation (Liu et al., 2008; Frick et al., 2010b; Boulware et al., 2013), very little is known regarding the role of GPER in hippocampal memory consolidation.

This gap in our knowledge is important to address because manipulating GPER could provide the memory-enhancing effects of intracellular ER activation without cancerous side effects, as ERα and ERβ activation are implicated in certain types of cancer (Deroo and Korach, 2006; Burns and Korach, 2012). Whereas nuclear

ERα and ERβ expression increased or stayed constant during breast cancer progression (Filardo et al., 2006), GPER expression decreased, and other evidence suggests that GPER acts independently from ERα and ERβ in cancer cell lines (Filardo and Thomas, 2012). Furthermore, GPER activation suppresses cell proliferation in ovarian cancer cell lines (Ignatov et al., 2013). Therefore, understanding the role of GPER in estrogen signaling may help resolve some of the controversies related to estrogen's involvement in regulating both cognitive function and certain types of cancer. Moreover, better understanding of GPER function could also provide important opportunities for the development of new therapies that would provide the cognitive benefits of estrogens while limiting potentially dangerous side effects.

Hippocampus

The hippocampus is one of the most researched structures in the brain. It is a bilateral medial temporal lobe structure that plays a central role in the functioning of the limbic system, due to its connections with the temporal cortex (e.g., entorhinal, perirhinal, and parahippocampal cortices), septum, and amygdala (Arushanyan and Beier, 2008). The primary cell type within the hippocampus is the pyramidal neuron, which are organized into a form of three-layered cortical tissue. The hippocampus in subdivided into several subregions, denoted as CA1, CA2, CA3, and dentate gyrus, based on morphologic and functional composition. Although the specific functions of the hippocampus remain subject to debate, most investigators agree that the hippocampus plays a critical role in learning and memory.

The famous case study of patient H.M, firstly published by Brenda Milner, first suggested the critical importance of the hippocampus for memory formation

(Scoville and Milner, 1957). After surgery of a bilateral medial temporal lobectomy, H.M. had severely impaired memory, although not all types of memory were affected. H.M. experienced severely impaired declarative memory; H.M. had severe anterograde amnesia as well as partial retrograde amnesia, on the other hand, His nondeclarative memory and short-term memory was preserved (Corkin et al., 1997). Therefore, H.M. study demonstrated the organization of memory in the brain, long term memory and immediate memory and the findings from H.M. motivated the efforts to study the neurobiological mechanisms underlying memory formation in animal models, such as monkey and rodent models (Squire, 2009). Two of the most well-known functions of the hippocampus are the generation of cognitive maps for use in spatial navigation and regulating episodic memory processes (Smith and Mizumori, 2006). Analysis of neuronal activity, by recordings of single neurons in the hippocampus, revealed that the hippocampus is involved in spatial navigation, as well as other abilities including detecting speed and direction of movement, match or non-match detection, and olfactory discrimination (Holscher, 2003).

To test functioning of hippocampus in rodent models, many behavioral tests have been established. For example, spatial learning and memory can be evaluated using several different methods, including the Morris water maze, Barnes maze, radial arm maze, T-maze, and Y-maze (Yuede et al., 2007). Our laboratory uses object recognition and object placement tasks to test hippocampal- dependent object recognition memory and spatial memory because these one-trail tasks allow us to link memory consolidation with rapid molecular events within the hippocampus. Moreover, these tasks can be conducted using the same apparatus and training procedures, permitting observation of multiple forms of hippocampal memory under similar testing conditions.

Object recognition (OR) and object placement (OP)

OR and OP have ben used extensively to examine hippocampal memory in rodents because they are sensitive to numerous factors, including hormones, aging, and drug treatments (Tuscher et al., 2014). Although the tasks can be run with anywhere from 2-6 objects, most protocols for rodents typically require them to explore two identical objects in a testing arena. During the training phase, these two objects are usually identical. For object recognition, memory is tested after a delay by allowing subjects to explore one familiar object that is identical to the training objects and one novel object. Mice who remember the familiar object will spend more time than chance exploring the novel object. For object placement, memory is tested by moving one of the familiar training objects to a new location in the arena. Mice who remember the training object locations will spend more time than chance exploring the moved object.

Both OR and OP are well suited for investigating hormonal regulation of hippocampal memory because these tasks take advantage of rodent's instinct, attraction to novel stimuli, without other potential variables influencing motivation. For example, the Morris water maze involves the stress of submersion in water and dry land mazes like the radial arm maze, T-maze, and Y-maze involve the stress of nutrient restriction. These stressors can induce physiological changes, including hormone level changes that can differ between males and females (ter Horst et al., 2012). In contrast, OR and OP involve only the subject’s own intrinsic motivation to explore; it uses no nutrient restriction, provides no rewards, and it does not place subjects in an uncomfortable stressful environment (i.e., no water submersion, shock, or exposure to bright light). Therefore, OR and OP are ideal behavior tasks for

studying the effects of hormone treatment on learning and memory. Despite some controversy surrounding the role of the hippocampus in object recognition, several studies demonstrate the importance of the hippocampus in regulating object recognition (Clark et al., 2000; Broadbent et al., 2004). In fact, one recent study suggests that inactivation of a very small portion of the total hippocampus can impair object recognition memory (Cohen et al., 2013). Furthermore, ovariectomy impairs memory in both OR and OP (Wallace et al., 2006), and as will be demonstrated below, estrogen treatment enhances OR and OP memory consolidation.

Estrogen effects on the hippocampus

Estrogens are a class of sex steroid hormones that are synthesized primarily within the ovaries and placenta, although smaller amounts of estrogens are also synthesized in non-gonadal organs such as the heart, liver, bone, and muscle (Cui et al., 2013). Estrogens influence many physiological processes via estrogen receptors (ERs), including reproduction, bone integrity, cognition, and parenting behaviors. The three major forms of estrogens are estrone (E_1), estradiol (E_2), and estriol (E_3). Of these, E_2 is most potent and biologically active. E_2 levels in the rat hippocampus are higher than in serum (Hojo et al., 2004), implying an important role of estrogens in the hippocampus. The earliest findings to demonstrate that E_2 regulates hippocampal function showed that dendritic spine density in the CA1 region in the female rat hippocampus is elevated when estrogen levels are their highest during the estrous cycle and that E_2 treatment reverses an ovariectomy-induced decrease in CA1 spine synapse density (Gould et al., 1990; Woolley et al., 1990). Exogenous E_2 also increases hippocampal neurogenesis and enhances various forms of hippocampal synaptic plasticity, including long-term potentiation (Foy et al., 1999; McClure et al.,

2013). In general, estrogens have been shown to enhance hippocampal memory in menopausal women and female rodents (Duff and Hampson, 2000; Frick, 2009). Many studies have demonstrated that exogenous E_2 administration enhances hippocampal memory using a variety of tasks, including the Morris water maze, radial arm maze, and T-maze (Bimonte and Denenberg, 1999; Daniel and Dohanich, 2001; Wide et al., 2004; Bohacek and Daniel, 2007). As discussed below and in our recent review (Tuscher et al., 2014), E_2 facilitates memory consolidation in the OR and OP tasks as well. However, the molecular mechanisms underlying this enhancement are not well understood.

Using an ovariectomized mouse model, our laboratory previously demonstrated that post-training bilateral infusion of E_2 into the dorsal hippocampus (DH) enhances hippocampal-dependent memory consolidation in the OR task (Fernandez et al., 2008). Other work from our lab has shown that DH E_2 infusions also enhance hippocampal-dependent spatial memory in OP (Boulware et al., 2013). Although the role of the hippocampus in OR has been subject to debate (Gervais et al., 2013), DH lesions or inactivations demonstrate that the DH is essential for object recognition memory consolidation in rats and mice (Clark et al., 2000; Baker and Kim, 2002; Fernandez et al., 2008; Cohen et al., 2013). In our laboratory, E_2 is infused immediately after training in OR and OP, rather than before training, to pinpoint E_2's effects on memory consolidation without affecting motivation, anxiety, or encoding during training. Infusion of E_2 three hours after training does not enhance memory consolidation (Fernandez et al., 2008), suggesting that E_2-induced hippocampal memory consolidation occurs within three hours of training. Vehicle-infused young ovariectomized mice show a significant preference for the novel object 24 hr, but not 48 hr, after OR training. However, E_2-infused mice exhibit enhanced memory 48 hr

after OR training, as indicated by their spending significantly more time than chance with the novel object. For the OP task, vehicle-infused young ovariectomized mice show a significant preference for the moved object 4 hr, but not 24 hr, after OP training. However, E_2-infused mice exhibit a significant preference for the moved object 24 hr after OP training. Thus, to test the memory-enhancing effects of drugs, we use a 48-hr retention delay for OR and a 24-hr delay for OP. To test the memory-impairing effects of drugs, we use a 24-hr delay for OR and a 4-hr delay for OP.

Our laboratory has extensively studied the molecular mechanisms through which E_2 affects hippocampal memory (Harburger et al., 2007; Lewis et al., 2008; Pechenino and Frick, 2009; Fan et al., 2010; Frick et al., 2010a; Zhao et al., 2010; Zhao et al., 2012; Boulware et al., 2013; Fortress et al., 2013b; Fortress et al., 2013a; Fortress et al., 2014). In particular, we have shown repeatedly that phosphorylation of the p42 isoform of the cell signaling kinase extracellular signal regulated kinase (ERK) in the DH is necessary for E_2 to enhance OR memory (Fernandez et al., 2008; Fan et al., 2010; Zhao et al., 2010; Zhao et al., 2012). This activation is observed as early as five minutes after DH infusion of E_2. The importance of ERK in mediating the mnemonic effects of E_2 was underscored by other work from our laboratory showing that rapid activation of the p42 isoform of ERK (p42-ERK) is required for histone acetylation alterations that promote the transcriptional events that enhance memory consolidation (Zhao et al., 2010). Although this work sheds light on the intracellular events that underlie the memory-enhancing effects of E_2, the ERs that mediate these effects have remained somewhat of a mystery.

Estrogen receptors

Two types of ERs, intracellular ERs (ERα and ERβ) and membrane ERs (e.g.,

GPER, ER-X) likely mediate the memory-enhancing effects of E_2. The intracellular ERs, ERα and ERβ, have been cloned and are found in several brain regions including the hippocampus of the nuclei, dendritic spines, and axon terminals of pyramidal neurons and interneurons (Milner et al., 2001; Milner et al., 2005). 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 initiate gene transcription (Cheskis et al., 2007). This so-called classic nuclear action of estrogens is considered somewhat slow because the cellular effects can take hours to be observed. However, ERα and ERβ can also activate hippocampal cell signaling cascades within minutes, suggesting an alternative mechanism of action. Such rapid effects have been termed ″non-classical" mechanisms. One established non-classical mechanism regulating involves interactions between ERα and ERβ and metabotropic glutamate receptors (mGluR) to stimulate the phosphorylation of the transcription factor cAMP response element-binding protein (CREB) (Boulware et al., 2005). Although studies using ERα and ERβ knockout mice have suggested that the effects of E_2 on hippocampal memory are dependent on ERβ, but not ERα (Liu et al., 2008; Walf et al., 2008), potential compensatory mechanisms after gene knockout throughout early development make it difficult to pinpoint the roles of each intracellular ER. Therefore, ERα-selective and ERβ-selective agonists have been developed to differentiate the role of each ER to memory formation.

Recently, our laboratory used ER agonists to show that the intracellular estrogen receptors ERα and ERβ can mediate the E_2-induced enhancement of object recognition and object placement memory consolidation (Boulware et al., 2013). Specifically, bilateral infusion of propyl pyrazole triol (PPT, ERα agonist) or

diarylpropionitrile (DPN, ERβ agonist) into the DH immediately after OR or OP testing enhanced object recognition and object placement memory consolidation. Like E_2, both PPT and DPN increased phosphorylation of the p42, but not the p44, isoform of ERK 5 minutes after infusion, and this activation was necessary for PPT and DPN to enhance memory. Because ERα and ERβ are not integral membrane proteins, it is unlikely that they activate ERK on their own. Instead, we found that both receptors must interact mGluR1 to rapidly activate ERK signaling and enhance memory consolidation (Boulware et al., 2013).

As an alternative to intracellular receptors, E_2 may regulate memory by binding to membrane ERs (mERs). The existence of specific mERs has been the subject of intensive debate in recent years because they have yet to be cloned. Candidate mERs include G-protein coupled estrogen receptor 1 (GPER), ER-X, and Gq-mER. Despite the uncertainty surrounding the identity of the mERs, these receptors can be examined generally using bovine serum albumin (BSA)-conjugated E_2 (BSA-E_2), which is membrane impermeable (Taguchi et al., 2004). Unlike E_2, BSA-E_2 does not activate estrogen responsive gene transcription (Watters et al., 1997). Instead, BSA-E_2 rapidly activates calcium signaling and ERK phosphorylation *in vitro* and *in vivo* (Carrer et al., 2003; Wu et al., 2011). In addition, our laboratory has found that infusion of BSA-E_2 into the DH of ovariectomized female mice enhances OR memory consolidation in an ERK-dependent manner (Fernandez et al., 2008). These effects were not blocked by an intracellular ER antagonist (ICI 182,780) (Fernandez et al., 2008), suggesting that mER activation can influence memory and ERK activation independently of intracellular ERs. Although informative, studies using BSA-E_2 do not provide information about which mERs are involved. Given the difficulty of identifying these ERs, it is therefore, challenging to target them

pharmacologically or genetically. However, the availability of agonist and antagonist drugs for the recently named GPER had led to an increasing number of studies aimed at understanding the role of this putative mER in memory formation.

G-Protein Coupled Estrogen Receptor 1 (GPER)

GPER is a G-protein coupled receptor, previously known as the orphan GPCR called GPR30 (Funakoshi et al., 2006). Although has been considerable debate about whether GPER is, indeed, a mER (Langer et al., 2010), there was sufficient evidence from peripheral tissues that the receptor's name was officially changed from GPR30 to GPER. GPER is expressed at high levels in the brain, including the hippocampus (Brailoiu et al., 2007). Within the hippocampus, GPER is localized within dendritic spines of excitatory synapses and peri-synaptic regions in CA1 hippocampal neurons (Akama et al., 2013; Srivastava and Evans, 2013). GPER is a seven transmembrane domain (7TMD) receptor that includes the heterotrimeric G protein subunits Gαβγ (Filardo and Thomas, 2005). The Gα protein is involved in regulating ion channels and membrane-associated enzymes, whereas the Gβγ-subunit plays a role in activating protein kinase cascades (Luttrell et al., 1999; Filardo and Thomas, 2005). Importantly, G proteins provide signaling mechanisms critical for the regulation of different mitogen-activated protein kinase (MAPK) (Goldsmith and Dhanasekaran, 2007). Some studies suggest that activation of the SRC-like tyrosine kinase downstream of GPER can promote the induction of the MAPK pathway (Maggiolini and Picard, 2010), and that both E_2 and the GPER agonist G-1 increase ERK phosphorylation in pancreatic beta cells (Sharma and Prossnitz, 2011). However, other studies indicate that activation of GPER does not induce ERK phosphorylation in human vascular smooth muscle cells (Ortmann et al., 2011) and that ERK

inhibition has no effect on the ability of G-1 to induce DNA synthesis in human epithelial cells (Holm et al., 2011). Several other downstream targets of GPER have been characterized, including a SRC-like tyrosine kinase (Quinn et al., 2009), PKA via cAMP (Thomas et al., 2005), PI3K/Akt (Maggiolini and Picard, 2010), and the Notch signaling pathway (Ruiz-Palmero et al., 2011).

Interestingly, some reports have demonstrated that ERα localized at the membrane interacts directly with various G-proteins (Wyckoff et al., 2001; Kumar et al., 2007). However, potential interaction between GPER and intracellular ERs has not been examined. As mentioned above, whether GPER is, in fact, an estrogen receptor has been a matter of heated debate. Some investigators insist that GPER is not a true ER, but potentially has a collaborative role in mediating the biological actions of estrogens (Levin, 2009). Although this issue has not yet been resolved for neural tissue, evidence in peripheral tissues suggests that GPER binds E_2 with a high affinity (Thomas et al., 2005), prompting the name change from GPR30 to GPER.

The contribution of GPER to hippocampal memory formation is not well established. However, some pharmacological studies have examined the role of GPER in memory processes using systemic injections of the GPER agonist, G-1, and antagonist, G-15. G-1 is a selective agonist for GPER that does not bind ERα and ERβ at concentrations up to 10 μM *in vitro* (Bologa et al., 2006; Blasko et al., 2009) and G-15 is selective antagonist for GPER that also does not bind to ERα and ERβ at concentrations up to 10 μM *in vitro* using COS7 cells (fibroblast-like cells) (Dennis et al., 2009). One recent study showed that chronic systemic treatment with G-1 mimics the beneficial effects of E_2 on spatial working memory in young female rats (Hammond et al., 2009). In contrast to G-1, systemic treatment with G-15 impairs

spatial working memory in young female rats (Hammond and Gibbs, 2011). Although these studies suggest that GPER regulates hippocampal memory, their use of systemic injections do not permit definitive conclusions about the role of hippocampal GPER in memory formation. To address this issue, this thesis employed direct DH infusions of G-1 and G-15 to pinpoint the role of hippocampal GPER in memory consolidation.

c-Jun N-terminal kinase (JNK)

G-proteins like GPER can activate numerous cell-signaling cascades. As will be discussed below, our results led us to examine cascades other than ERK, including the JNK signaling pathway. Like ERK, JNK belongs to the mitogen-activated protein kinase (MAPK) family and has a kinase signaling cascade structure in which mitogen-activated protein kinase kinase (MKK) 4 and MKK7 are direct activators of JNK (Haeusgen et al., 2009). 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 and Borsello, 2012).

JNK has most often been studied in the context of cellular stress and apoptosis related to heat shock or DNA damage (Kyriakis and Avruch, 2001; Reinecke et al., 2013). In the nervous system, JNK plays an important role in synaptic plasticity, neuronal regeneration, and brain development (Tararuk et al., 2006; Waetzig et al., 2006). Evidence has also suggested that JNK activity is involved in the regulation of the post-synaptic density protein called post-synaptic density-95 (PSD-95) (Kim et al., 2007). Interactions between GPER and PSD-95 have been identified in hippocampal dendritic spines (Akama et al., 2013), suggesting a possible link between JNK and GPER at the synaptic membrane. Much less is known, however,

about JNK's role in learning and memory. Studies of JNK inhibitor-treated mice suggest a facilitative role of JNK activation in long-term inhibitory avoidance memory and neuroprotective effects in hippocampal neurons (Bevilaqua et al., 2007; Carboni et al., 2008). In addition, a study using JNK1-deficient (JNK1-/-) mice found that JNK1 may play a crucial role in short-term synaptic plasticity and mGluR-dependent long-term depression (Li et al., 2007). However, other data indicate that JNK inhibition in the hippocampus enhances short-term memory (Bevilaqua et al., 2003), suggesting that JNK may also negatively regulate memory. Therefore, the role of JNK signaling in hippocampal memory formation is unclear. Furthermore, nothing is known about whether JNK signaling is involved estrogenic regulation of the hippocampus or memory. As such, the role of JNK in mediating the effects of E_2 or GPER on memory was of interest in this thesis.

Aims

Given the uncertainty surrounding the role of the putative mER GPER in memory formation, the primary goals of this thesis were to pinpoint the role of GPER in regulating hippocampal object recognition and spatial memory consolidation and determine the molecular mechanisms underlying this regulation. To achieve these goals, we conducted a series of studies in which we infused a GPER agonist or antagonist directly into the DH of ovariectomized mice immediately after training in the OR and OP tasks.. We found that GPER regulates both object recognition and spatial memory consolidation, but that these effects were dependent on JNK, but not ERK activation in the DH. Next, we found that the memory-enhancing effects of E_2 were not dependent on JNK or GPER activation in the DH. Collectively, these data suggest that GPER enhances hippocampal memory consolidation by activating

different cell signaling cascades than E_2. As such, GPER does not appear to function as a mER in the hippocampus.

Materials and Methods

Subjects

Subjects were female C57BL/6 mice (8-10 weeks of age) purchased from Taconic Biosciences (Cambridge City, IN). After surgery, mice were singly housed in a room with a 12-hour light/dark cycle, and were allowed *ad libitum* access to food and water. All behavioral testing was performed between 9 am and 6 pm in a quiet room with dim lights. All procedures were approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee, and followed policies set forth by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Surgery

At least one week prior to behavioral testing, mice were bilaterally ovariectomized and implanted with chronic indwelling guide cannuale within the same surgical session as previously described (Boulware et al., 2013; Fortress et al., 2013b; Fortress et al., 2014). Mice were anesthetized with isoflurane gas (2% isoflurane in 100% oxygen) and secured in a stereotaxic apparatus (Kopf Instruments). Following ovariectomy, mice were implanted with guide cannulae (22 gauge, C232G, Plastics One) into the DH (-1.7 mm AP, ±1.5 mm ML, -2.3 mm DV) or DH and dorsal third ventricle (intracerebroventricular (ICV); -0.9 mm AP, ±0.0 mm ML, -2.3 mm DV) as previously performed (Boulware et al., 2013; Fortress et al., 2013b; Fortress et al., 2014). Dummy cannulae (C232DC, Plastics One) were inserted into all guide cannulae to preserve patency of the guide cannulae. Cannulae were fixed to the skull with dental cement (Darby Dental Supply) that served to close the wound.

Mice were allowed 7 days to recover from surgery before the start of behavioral testing.

Drugs and infusions

During infusions, mice were gently restrained and dummy cannulae were replaced with an infusion cannula (C3131; DH: 28 gauge, extending 0.8 mm beyond the 1.5 mm guide; ICV: 28 gauge, extending 1.0mm beyond the 1.8 mm guide) attached to PE20 polyethylene tubing that was mounted on a 10 µl Hamilton syringe. Infusions were controlled by a microinfusion pump (KDS Legato 180; KD Scientific). All infusions were conducted immediately post-training at a rate of 0.5 µl/minute in the DH or 1 µl/2 minutes into the dorsal third ventricle as described previously (Boulware et al., 2013; Fortress et al., 2013b; Fortress et al., 2014). Infusion cannulae remained in place for 1 min after each infusion to prevent diffusion back up the cannula track. For studies in which E_2 or G-1 was administered in combination with G-15 or a cell-signaling inhibitor, the antagonist or cell-signaling inhibitor was first infused bilaterally into the DH and then E_2 or G-1 was infused ICV immediately afterwards. We routinely use this triple infusion protocol to prevent possible tissue damage from two DH infusions in rapid succession (Fernandez et al., 2008; Fan et al., 2010; Zhao et al., 2010; Zhao et al., 2012; Boulware et al., 2013; Fortress et al., 2013b). This protocol allows us to infuse estrogenic compounds adjacent to the DH while inhibiting receptor or cell-signaling activation directly within the DH.

G-1, 1-[4-(6-bromobenzo[1,3]dioxol-5yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta [c]quinolin-8-yl]-ethanone (Azano biotech) was dissolved in 16% dimethylsulfoxide (DMSO) and infused at doses of 2 or 4 ng/hemisphere into the DH or 8 ng ICV. G-1 is a selective agonist for GPER that does not bind ERα and ERβ at

concentrations up to 10 µM *in vitro* (Bologa et al., 2006; Blasko et al., 2009). 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 (Azano biotech) was dissolved in 2% DMSO and infused at doses of 1.85, 3.7, and 7.4 ng/hemisphere into the DH. G-15 is selective antagonist for GPER that also does not bind to ERα and ERβ at concentrations up to 10 µM *in vitro* (Dennis et al., 2009). The vehicle control for G-15 was 2% DMSO in 0.9% saline.

Cyclodextrin-encapsulated E_2 (Sigma-Aldrich) was dissolved in 0.9% saline and infused at doses of 5 µg/hemisphere into the DH or 10 µg ICV (Zhao et al., 2012; Boulware et al., 2013). The vehicle control for E_2 was 2-hydroxypropyl-β-cyclodextrin (HBC, Sigma-Aldrich), dissolved in 0.9% saline using the same amount of cyclodextrin as E_2 for infusions. The JNK inhibitor SP600125 (Anthra[1,9-cd]pyrazol-6(2H)-one, Sigma-Aldrich) was dissolved in 2% DMSO and infused at doses of 0.11, 0.55, and 2.75 ng/hemisphere into the DH. SP600125 is a selective inhibitor for JNK that does not affect ERK and p38 at concentrations below 10 µM (Bennett et al., 2001). The vehicle control for SP600125 was 2% DMSO in 0.9% saline. The MEK inhibitor U0126 (1,4-diamino-2,3-dicyano-1,4-bis (o-aminophenylmercapto) butadiene, Promega) was dissolved in 25% DMSO and infused at a dose of 0.5 µg/hemisphere into the DH. This dose does not impair OR and OP memory by itself (Fernandez et al., 2008; Boulware et al., 2013), and therefore, any effects of this drug in combination with E_2 or G-1 cannot be attributed to a general memory impairing effect of this compound. The vehicle control for U0126 was 25% DMSO in 0.9% saline.

Object recognition and object placement

OR and OP were conducted to examine hippocampus-dependent object recognition and spatial memory. Both tasks have been shown to involve dorsal hippocampal function (Baker and Kim, 2002; Luine et al., 2003; Frye et al., 2007; Cohen et al., 2013) and are sensitive to E_2 treatment (Gresack and Frick, 2006; Zhao et al., 2010). Before the start of behavioral training, mice were handled (1 min/day) for three days to acclimate them to the experimenters. They were also familiarized with objects by placing a small Lego not used during testing in their home cage. At the start of training, mice were habituated to the empty white arena (width, 60 cm; length, 60 cm; height, 47 cm) by allowing them to explore for five min/day for two consecutive days. On third day, mice were habituated for two minutes in the arena, and then placed in a holding cage while two identical objects were placed near the northwest and northeast corners of the arena. Mice were then returned to the arena and allowed to freely explore the objects until they accumulated 30 s of investigation. Immediately after this training, mice were infused and then returned to their home cage. After 24 or 48 h, memory was tested by allowing mice to accumulate 30 s exploring a novel object and an object identical to the familiar training objects. Time spent with the objects was recorded using by ANYmaze tracking software (Stoelting). Because mice inherently prefer novelty, mice who remember the familiar object spend more time investigating the novel object than chance (15 s). Vehicle-infused mice do not remember the familiar object 48 h after training (Gresack et al., 2007), so we used this delay to test the memory enhancing effects of E_2 and G-1. However, vehicle-infused females do remember the familiar object 24 h after training (Gresack et al., 2007), so this shorter delay was used to test for potential memory impairing effects of G-15 and cell-signaling inhibitors.

The OP task used the same apparatus and general procedure as OR, but instead of substituting a novel object for a training object during testing, one familiar object was moved to the Southeast or Southwest corner of the testing arena. Because vehicle-infused females remember the original object placement after 4 h, but not 24 h(Boulware et al., 2013), we used the 24-h delay to test memory enhancing effects of E_2 and G-1 and the 4-h delay to test memory impairing effects of G-15 and cell-signaling inhibitors. Two weeks separated OR and OP testing to allow acute effects of the drug infusions to dissipate prior to the next infusion (n = 6-12/group).

Western blotting

Western blotting was performed as described previously (Fernandez et al., 2008; Boulware et al., 2013). To determine the effects of G-1 on DH cell signaling, mice were cervically dislocated and decapitated, and the dorsal hippocampus was dissected bilaterally 5, 15, or 30 min after infusion and stored at -80°C until homogenization. To determine the effects of E_2, GPER compounds, and cell-signaling inhibitors on DH cell signaling, the DH was dissected bilaterally 5 min after infusion. DH tissues were resuspended 50 µl/mg in lysis buffer and homogenized by sonication (Branson Sonifier 250). Proteins were then electrophoresed on 10% Tris-HCl precast gels (Bio-Rad) and transferred to PVDF membranes (Bio-Rad). Western blots were blocked with 5% skim milk and incubated with primary antibodies (phospho-ERK, phospho-Akt, phospho-PI3K, phospho-JNK, and phospho-ATF2, 1:1000; Cell Signaling Technology) overnight. Blots were then incubated with the appropriate HRP-conjugated secondary antibody (1:5000; Cell Signaling), and developed using West Dura chemiluminescent substrate (Pierce). A ChemiDocMP gel imager (Bio-Rad) was used for signal detection of protein expression. Densitometry was performed

using Carestream Molecular Imaging Software (Carestream Healthcare). Blots then were stripped with 0.2M NaOH and incubated with antibodies (total-ERK, total-Akt, total-PI3K, and total-JNK, 1:1000; β-actin, 1:5000; Cell Signaling Technology) for protein normalization. Data were represented as immunoreactivity percent of vehicle controls. Treatment effects were measured within single gels (n = 5-8/group).

Statistics

For OR and OP data, one-sample *t*-tests were conducted using SPSS (IBM, Armonk, NY) to determine if each group spent more time than chance (15 s) exploring the novel or moved object (Gresack and Frick, 2003). Western blotting data were analyzed in GraphPad Prism 6 (La Jolla, CA) using one-way ANOVA followed by Fisher's LSD posthoc tests and selected *t*-tests . Significance was determined at $p < 0.05$.

Results

GPER regulates hippocampal memory consolidation

We first infused the GPER agonist G-1 into the DH to determine if activation of GPER in the DH enhances object recognition and object placement memory consolidation in a manner similar to E_2. Mice received bilateral DH infusion of vehicle (16% DMSO) or on of two doses of G-1 (2 or 4 ng/hemisphere) immediately after OR training. Forty-eight hours later, mice infused with vehicle of 2 ng G-1 spent no more time with the novel object than chance (15 s). In contrast, mice infused with 4 ng/hemisphere of G-1 spent more time exploring the novel object than chance ($t_{(8)}$ = 2.56, p = 0.03; Fig.1A), suggesting that 4 ng G-1 enhanced object recognition memory consolidation. Two weeks after OR testing, mice were trained in OP and then were immediately infused with vehicle, 2 ng G-1, or 4 ng G-1. Twenty-four hours later, mice infused with vehicle or 2 ng G-1 did not exhibit a preference for the moved object. However, as in OR, mice receiving 4 ng/hemisphere of G-1 spent significantly more time than chance with the moved object ($t_{(9)}$ = 3.81, p = 0.004; Fig. 1B), demonstrating enhanced spatial memory consolidation.

Because these data suggest that activation of GPER facilitates hippocampal memory consolidation, we next examined effects of GPER antagonism on memory consolidation. Immediately after OR or OP training, mice received bilateral DH infusion of vehicle (2% DMSO) or one of three doses of G-15 (1.85, 3.7, or 7.4 ng/hemisphere). Mice receiving vehicle ($t_{(8)}$ = 3.52, p = 0.008) or 1.85 ng/hemisphere of G-15 ($t_{(7)}$ = 3.32, p = 0.013) showed preference for the novel object 24 h after OR training suggesting intact object recognition memory after treatment with a low dose of G-15. In contrast, mice receiving 3.7 ($t_{(8)}$ = 2.02, p = 0.08) or 7.4 ($t_{(6)}$ = 0.89, p = 0.41) ng/hemisphere of G-15 did not (Fig. 1C), suggesting that these doses impaired

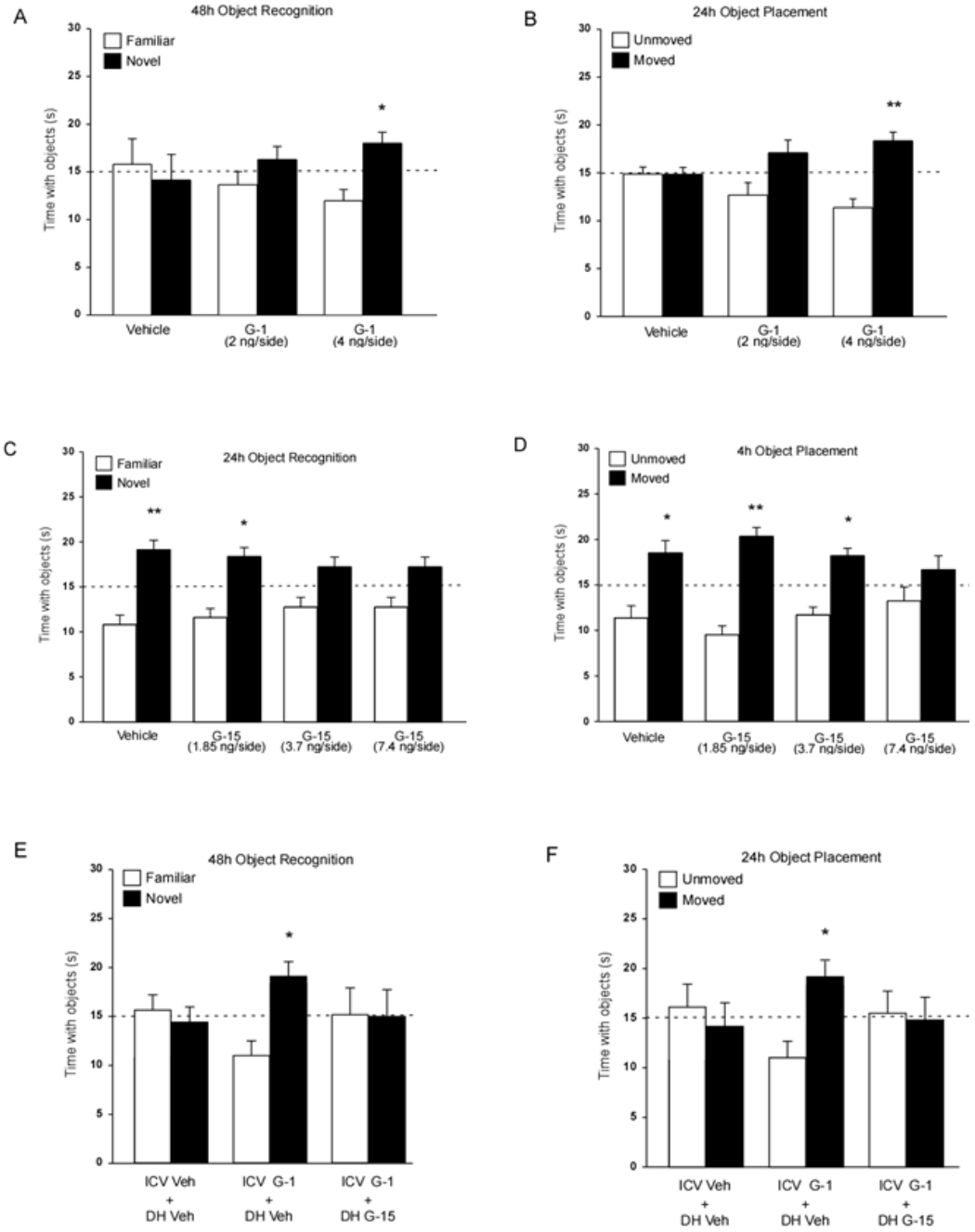

Figure 1. GPER activation enhances OR and OP memory consolidation. *A*, Mice receiving DH infusion of 4 ng/hemisphere G-1 (but not vehicle or 2 ng G-1) spent more time than chance (dashed line at 15 s) with the novel object 48 hr after training, indicating enhanced memory for the familiar object (n = 6-9/group). *B*, Similarly, mice infused with 4 ng G-1, but not vehicle or 2 ng G-1, spent significantly more time with the moved object than chance 24 h after OP training, indicating enhanced spatial memory (n = 9-10/group). *C*, Mice receiving 3.7 or 7.4 ng/hemisphere G-15 exhibited impaired OR memory consolidation 24 h after DH infusion, whereas mice receiving vehicle or 1.85 ng G-15 did not (n = 7-9/group). *D*, In OP, 7.4 ng G-15 impaired spatial memory consolidation 4 h after DH infusion, but no other dose of G-15 affected memory (n = 6-9/group). *E*, *F*, ICV infusion of 4 ng/hemisphere G-1 significantly enhanced OR (*E*) and OP (*F*) memory tested 48 h and 24 h after infusion, respectively (n = 8-11/group).. However, DH infusion of 1.85 ng/hemisphere G-15 abolished these effects, suggesting that activation of GPER is necessary for G-1-mediated hippocampal memory enhancement. Each bar represents the mean ± SEM time spent with the novel or moved object (*$p < 0.05$, **$p < 0.01$ relative to chance)..

object recognition memory consolidation. In OP, mice receiving DH infusion of vehicle ($t_{(8)} = 2.62$, $p = 0.03$), 1.85 ng G-15 ($t_{(7)} = 3.32$, $p = 0.013$), or 3.7 ng G-15 ($t_{(8)} = 2.02$, $p = 0.08$) spent significantly more time than chance (15 s) with the moved object, whereas mice infused with 7.4 ng G-15 did not (Fig. 1D). That only the high dose of G-15 impaired spatial memory consolidation suggests that spatial memory may be less sensitive to the effects of G-15 antagonism. that 1.85 ng/hemisphere of G-15 did not impair memory consolidation on its own and GPER inhibition impairs hippocampal memory although the sensitivity of task is a little bit different.

Finally, to confirm that G-15 acts as a GPER antagonist, we examined whether G-15 could block the memory-enhancing effects of G-1. To this end, we infused 8 ng G-1 into the dorsal third ventricle because bilateral infusion of 4 ng/hemisphere G-1 enhanced memory in both tasks. We also infused 1.85 ng/ hemisphere G-15 into the DH because this dose had no detrimental effects on memory in both tasks. Immediately after training in each task, mice received a DH infusion of vehicle (2% DMSO) or G-15 (1.85 ng/hemisphere) followed immediately by an ICV infusion of vehicle (16% DMSO) or G-1 (8 ng). OR and OP retention were tested 48 and 24 hours later, respectively. In both tasks, G-15 blocked the memory enhancing effects of G-1 (Fig. 1E,F). Only mice receiving G-1 + vehicle showed a significant preference for the novel object ($t_{(7)} = 2.68$, $p = 0.032$; Fig 1E) and moved object ($t_{(8)} = 2.46$, $p = 0.04$; Fig 1F). These results demonstrate that GPER activation is necessary for G-1 to enhance hippocampal memory consolidation in female mice, and suggest that GPER regulates both object recognition and spatial memory consolidation.

G-1 does not activate ERK or PI3K/Akt signaling in the DH

We have previously shown that the enhanced memory consolidation induced by DH infusion of E_2 or agonists of ERα and ERβ is dependent on DH p42 ERK phosphorylation (Fernandez et al., 2008; Boulware et al., 2013). To determine whether GPER also enhances memory by activating p42 ERK, we first measured the effects of GPER activation on ERK phosphorylation. Mice received bilateral DH infusion of 4 ng G-1 and the DH was dissected bilaterally 5, 15, or 30 min later.

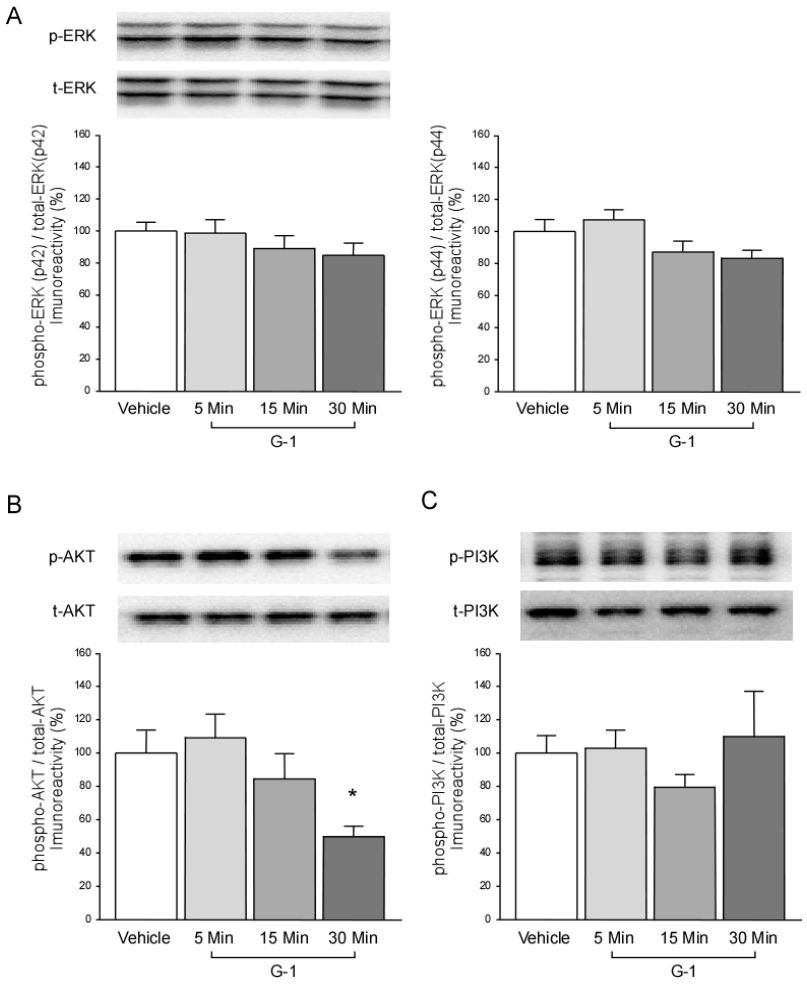

Figure 2. GPER does not activate the ERK or PI3K/Akt signaling pathways. *A*, G-1 (4 ng/hemisphere) infusion did not increase DH p42 and p44 ERK phosphorylation relative to vehicle 5, 15, or 30 min after DH infusion. *B*, G-1 infusion significantly reduced Akt phosphorylation levels in the DH 30 min after infusion. *C*, G-1 infusion did not alter PI3K phosphorylation relative to vehicle 5, 15, or 30 min after DH infusion. Each bar represents the mean ± SEM percent change from vehicle controls (*$p < 0.05$ relative to vehicle). Insets are representative Western blots. (n = 5/group).

In contrast to E_2(Fernandez et al., 2008; Boulware et al., 2013), G-1 infusion did not significantly increase levels of phospho-p42 ERK at any time point examined ($F_{(3,16)}$ = 0.72; Fig. 2A). G-1 also did not affect levels of phospho-p44 ERK ($F_{(3,16)}$ = 3.07; Fig. 2A). We then examined activation of the PI3K/Akt signaling pathway because we have previously demonstrated that activation of this signaling pathway is necessary for E_2 to activate ERK and enhance OR memory consolidation in young and middle-aged female mice (Fan et al., 2010; Fortress et al., 2013b). However, G-1 did not significantly increase levels of phospho-Akt ($F_{(3,16)}$ = 3.94, $p > 0.05$; Fig. 2B) or phospho-PI3K ($F_{(3,16)}$ = 0.68; $p > 0.05$; Fig. 2C). In fact, G-1 decreased levels of phospho-Akt 30 min after infusion (Fig. 2B). Collectively, these data show that GPER activation does not activate ERK or PI3K/Akt signaling in the DH and suggest that the effects of GPER activation on DH cell signaling are different from those of E_2 or ER agonists.

GPER activation leads to rapid JNK phosphorylation in the DH

We next investigated whether GPER activation could phosphorylate c-Jun N-terminal Kinase (JNK) in the DH. As a seven transmembrane domain receptor, GPER is comprised of heterotrimeric G protein subunits Gαβγ (Filardo and Thomas, 2005), and the Gβγ-subunit plays a role in activating protein kinase cascades such as ERK and JNK (Luttrell et al., 1999; Filardo and Thomas, 2005; Goldsmith and Dhanasekaran, 2007). Moreover, JNK is known to play an important role in synaptic plasticity, neuronal regeneration, and brain development (Tararuk et al., 2006; Waetzig et al., 2006). Therefore, we thought it possible that GPER might activate one or both of the two JNK isoforms (p46 and p54). Mice bilaterally infused into the DH with vehicle or 4 ng G-1 exhibited a significant increase in the phosphorylation of

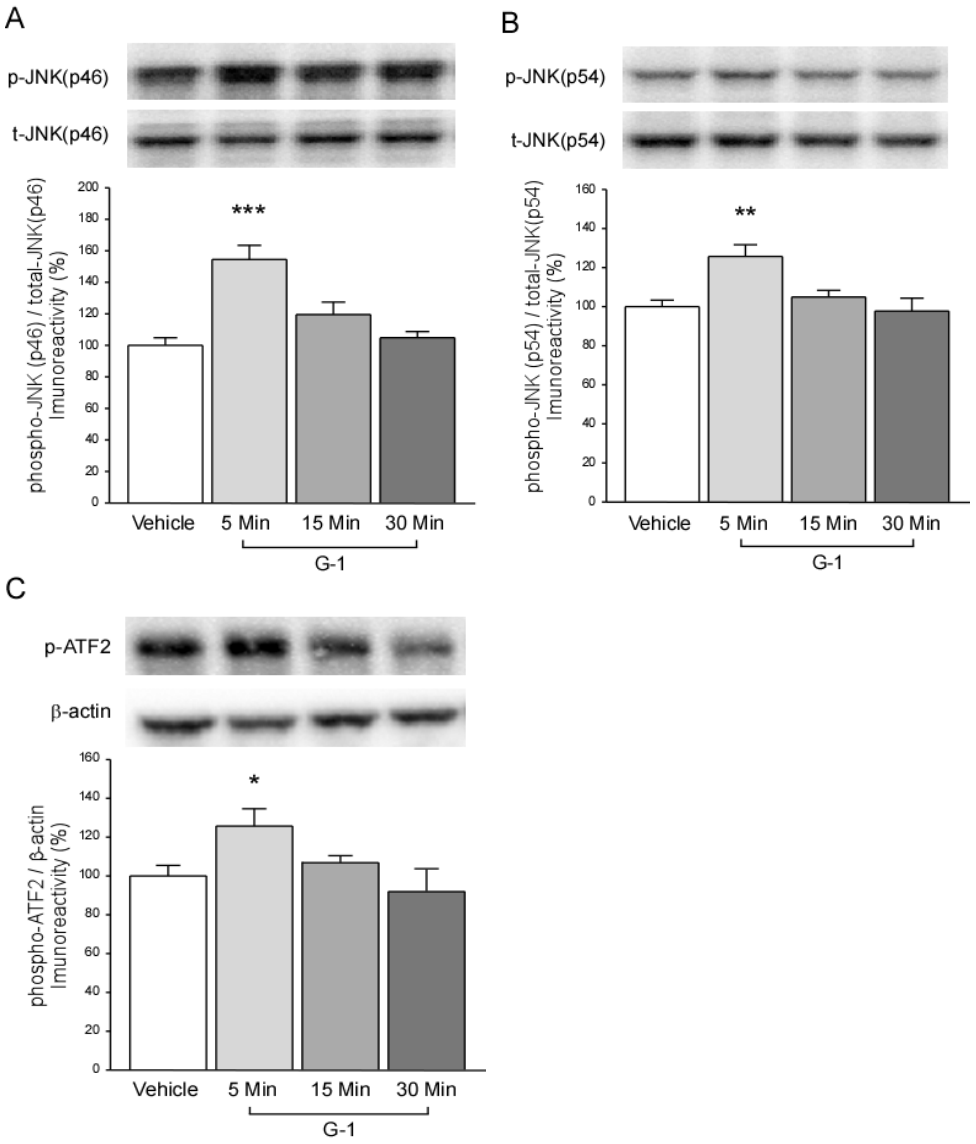

Figure 3. GPER activation increases JNK phosphorylation in the DH. *A,B*, DH infusion of G-1 (4 ng/hemisphere) significantly increased phosphorylation of the JNK p46 isoform (*A*) and p54 isoform (*B*) within 5 min. Levels returned to baseline 15 min later. *C*, Similarly, G-1 infusion significantly increased phosphorylation of the downstream JNK transcription factor ATF2 in the DH 5 min after infusion. (n = 5/group).

both the p46 ($F_{(3,16)} = 13.46$, $p < 0.001$; Fig. 3A) and p54 ($F_{(3,16)} = 6.335$, $p < 0.01$; Fig. 3B) isoforms of JNK 5 min after infusion. These effects were transient, as levels of both phosphorylated isoforms returned to baseline by 15 min after infusion. We next examined phosphorylation of the downstream JNK transcription factor called activating transcription factor 2 (ATF2) (Antoniou and Borsello, 2012). As with JNK, G-1 infusion significantly increased levels of, phospho-ATF2 5 min after DH infusion

($F_{(3,16)} = 3.03$, $p < 0.05$; Fig. 3C), and levels returned to baseline by 15 min after infusion.

To confirm that the G-1 mediated-JNK activation observed occurred via GPER activation, we next examined if G-15 could block the effects of G-1 on JNK

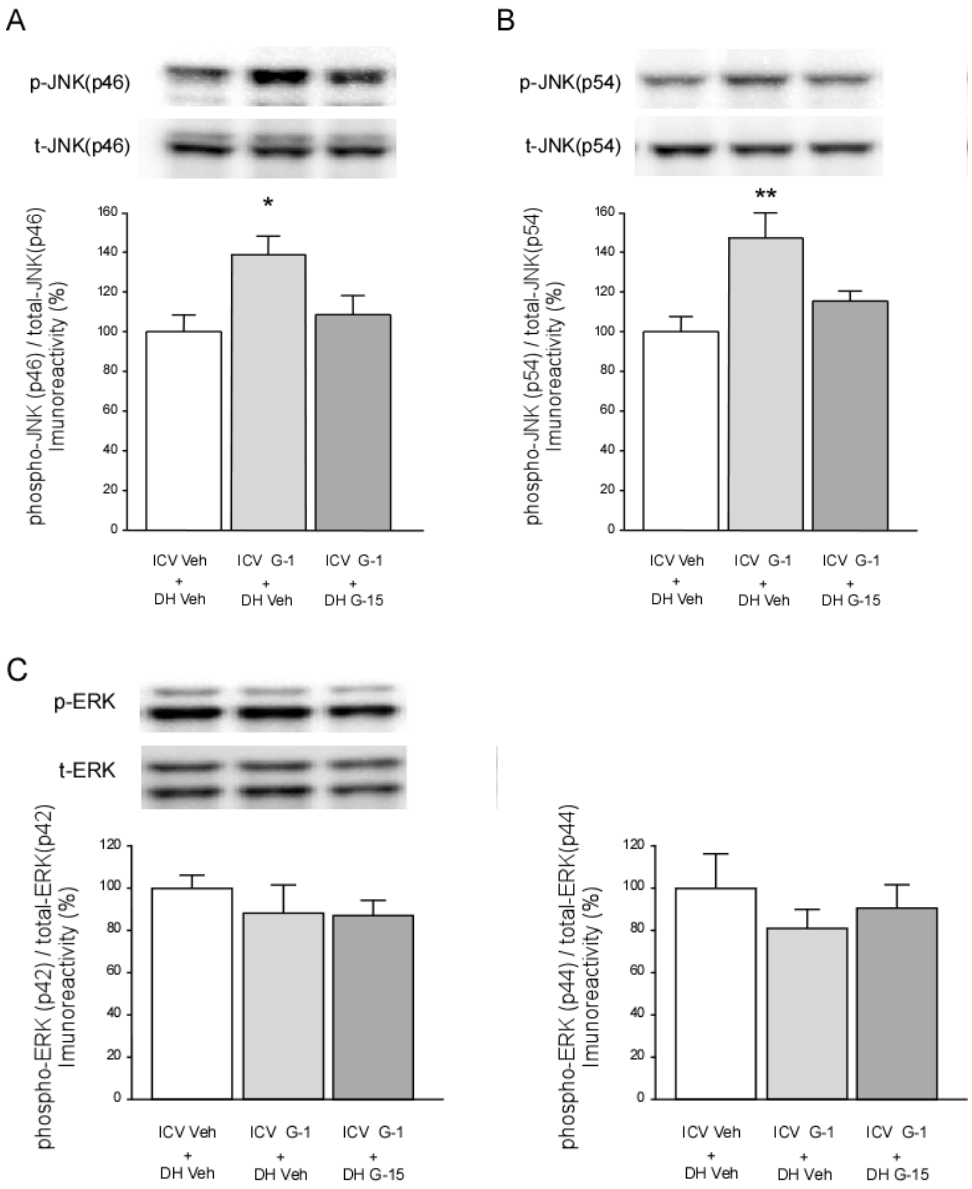

Figure 4. GPER antagonist blocks the G-1-mediated JNK phosphorylation in DH. *A,B,* ICV infusion of 8 ng G-1 significantly increased levels of phosphorylated p46 JNK (*A*) and p54 JNK (*B*) 5 min after infusion. However, these effects were abolished by DH infusion of G-15 indicating that GPER activation is necessary for G-1 to activate JNK signaling. *C*, Neither G-1 nor G-15 altered ERK phosphorylation. Each bar represents the mean ± SEM percent change from vehicle (*$p <$ 0.05, **$p < 0.01$, ***$p < 0.001$). Insets are representative Western blots. (n = 6/group).

activation. Mice received DH infusion of vehicle or G-15, and ICV infusion of vehicle or G-1. Consistent with the effects of DH G-1 infusion, ICV infusion of G-1 increased phosphorylation of both the p46 ($F_{(2,15)} = 4.97$, $p < 0.05$; Fig 4A) and p54 ($F_{(2,15)} = 7.89$, $p < 0.01$; Fig 4B) isoforms of JNK 5 min after infusion. Infusion of G-15 into the DH completely blocked these effects (Fig. 4A,B), suggesting that GPER activation induces JNK phosphorylation in the DH. In contrast, ICV and DH infusion of G-1 and G-15 did not significantly alter ERK phosphorylation (Fig. 4C).

Activation of JNK is necessary for GPER to influence hippocampal memory consolidation

Given the rapid activation of JNK by G-1, we next examined whether this activation is necessary for G-1 to enhance memory consolidation. To do so, we used the JNK activation inhibitor SP600125. We first needed to find a dose of SP600125 that did not block memory consolidation on its own. Therefore, we infused mice with vehicle (2% DMSO) or one of two doses of SP600125 (0.55 or 2.75 ng/hemisphere) immediately after OR or OP training. Mice receiving vehicle ($t_{(6)} = 3.27$, $p = 0.02$) or either dose of SP600125 (0.55 ng, $t_{(5)} = 2.7$, $p = 0.043$; 2.75 ng, $t_{(7)} = 3.46$, $p = 0.01$) spent significantly more time than chance with the novel object 24 h after OR training (Fig. 5A), suggesting that neither dose of SP600125 impaired OR memory consolidation. Similarly, mice infused with vehicle ($t_{(8)} = 3.87$, $p = 0.005$) or either dose of SP600125 (0.55 ng, $t_{(9)} = 3.45$, $p = 0.007$; 2.75 ng, $t_{(7)} = 3.7$, $p = 0.008$) spent significantly more time than chance with the moved object 4 h after OP training (Fig. 5B), indicating neither dose impaired OP memory consolidation. Because neither dose affected memory on its own, we selected the highest behaviorally ineffective dose of SP600125 (2.75 ng/hemisphere) for our remaining studies.

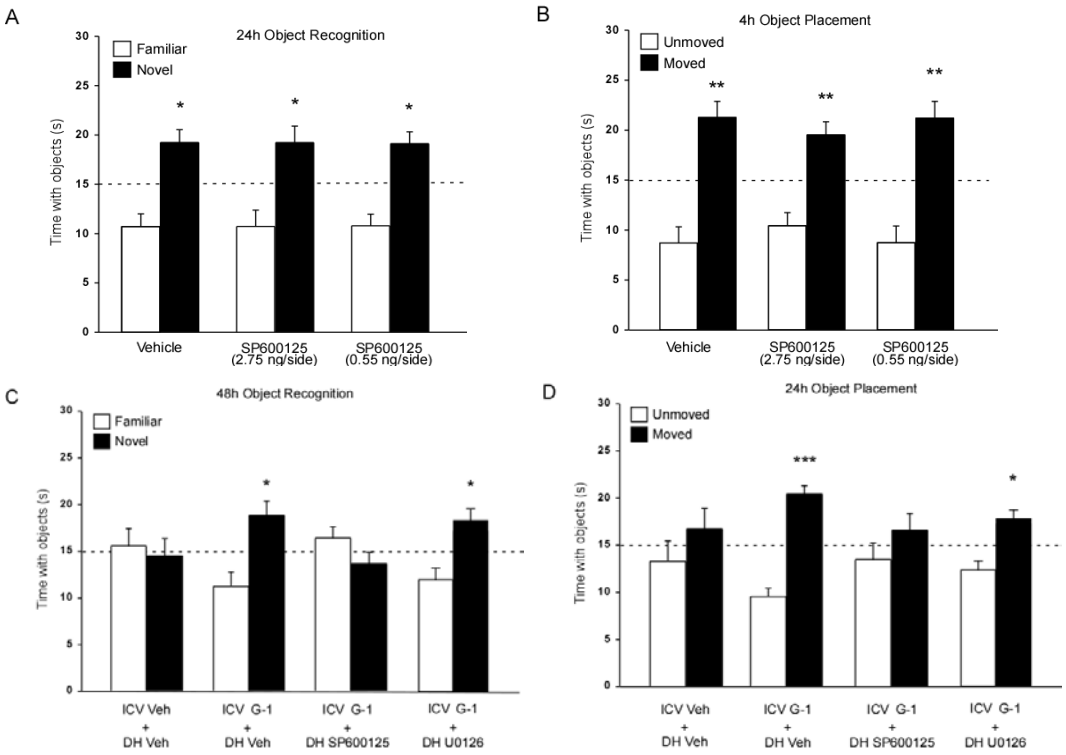

Figure 5. JNK inhibition blocks the GPER-mediated memory enhancement in DH. *A*, Mice receiving DH infusion of vehicle or the JNK inhibitor SP600125 (0.55 or 2.75 ng/hemisphere) spent significantly more time than chance with the novel object 24 hr after training, suggesting that neither dose of SP600125 impaired memory on its own (n = 6-8/group). *B*, Similarly, neither dose of SP600125 impaired OP memory tested 4 h after DH infusion (n = 6-10/group). *C, D*, Immediately after OR or OP training, mice received DH infusion of vehicle, SP600125 (1.85 ng/hemisphere), or U0126 (0.5 μg/hemisphere) followed by ICV infusion of vehicle or G-1 (8 ng). ICV infusion of G-1 significantly enhanced OR memory (*C*) and OP memory (*D*). SP600125 blocked these effects (*C, D*), but U0126 did not, suggesting that activation of JNK, but not ERK, is necessary for GPER-mediated hippocampal memory enhancement (n = 7-10/group).. 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).

To test whether activation of JNK or ERK was necessary for G-1 to enhance memory consolidation, we next infused mice with G-1 plus 2.75 ng SP600125 or the ERK activation inhibitor U0126 at a dose (0.5 μg/hemisphere) that has no effect on OR or OP on its own (Fernandez et al., 2008; Boulware et al., 2013). A new set of mice received DH infusion of vehicle (25% DMSO), 2.75 ng SP600125, or 0.5 μg U0126 and ICV infusion of vehicle (16% DMSO) or 8 ng G-1 immediately after OR

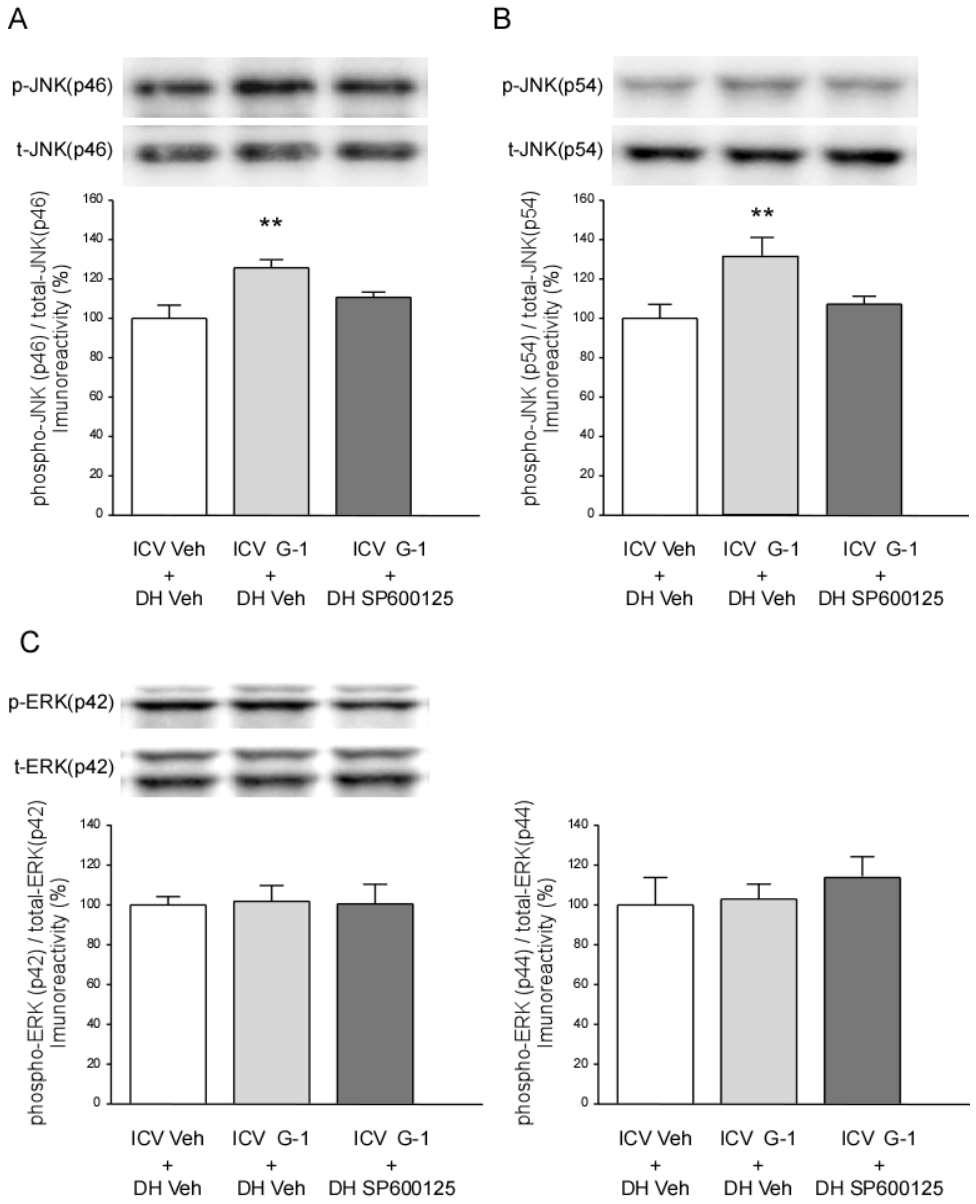

Figure 6. JNK inhibition blocks the GPER-mediated cell signaling in DH. *A, B*, ICV infusion of 8 ng G-1 increased phosphorylation of p46 JNK (*A*) and p54 JNK (*B*) 5 min later. These effects were blocked by DH SP600125 infusion. *C*, Neither G-1 nor SP600125 altered ERK phosphorylation (n = 7-8/group).

and OP training. Memory in OR and OP was tested 48 and 24 hours later, respectively. In both tasks, SP600125, but not U0126, blocked the memory-enhancing effects of G-1 (Fig. 5C,D). Mice receiving G-1 + vehicle showed a significant preference for the novel object ($t_{(9)} = 2.48$, $p = 0.04$) and moved object ($t_{(6)} = 6.37$, $p = 0.0007$), whereas mice receiving G-1 + SP600125 did not (novel object, $t_{(8)} = 1.16$, $p = 0.28$; moved

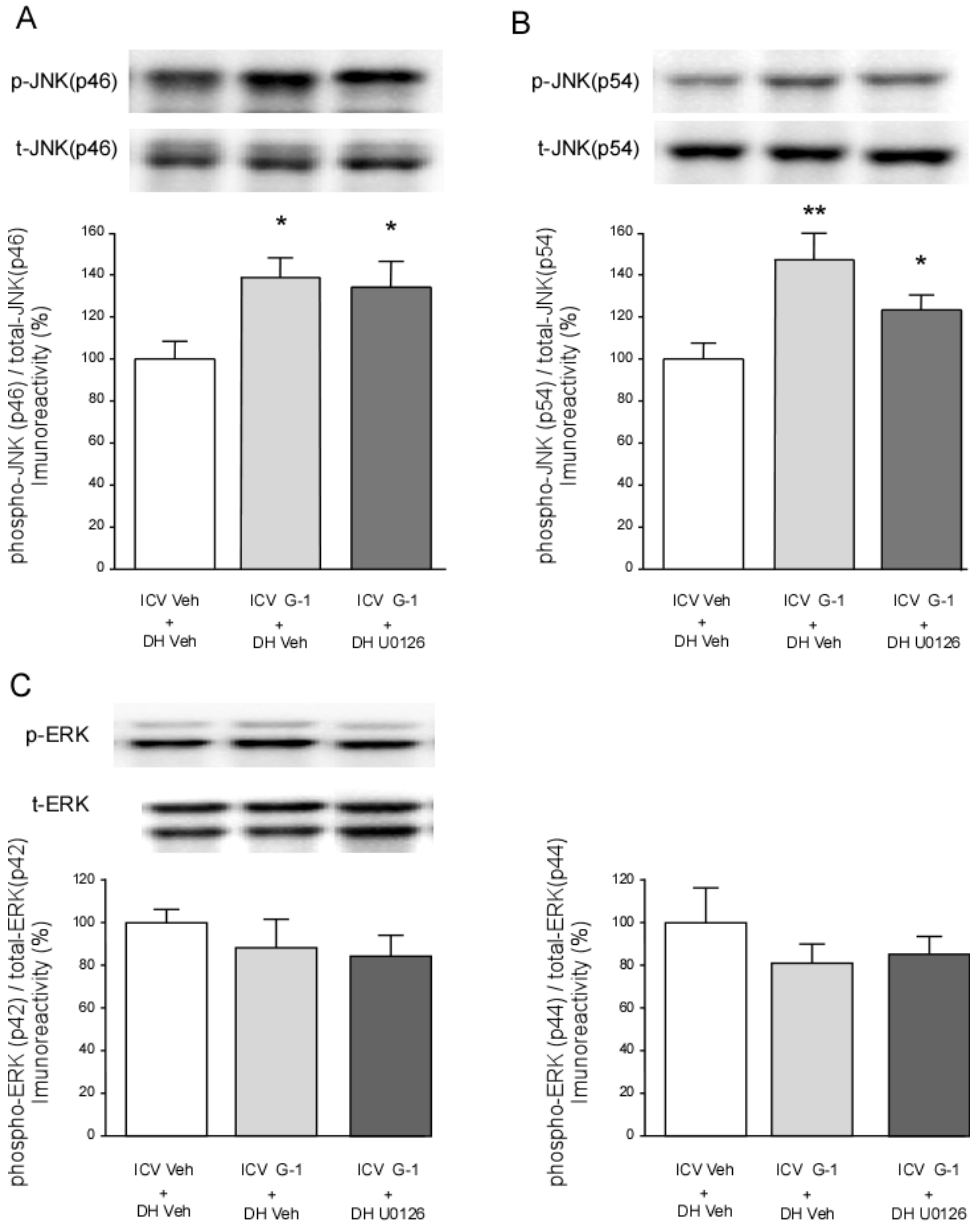

Figure 7. ERK inhibition does not alter the GPER-mediated cell signaling in DH. *A*, *B*, The increase in p46 (*A*) and p54 (*B*) phosphorylation induced by ICV infusion of 8 ng G-1 was not blocked by DH U0126 infusion. *C*, Neither G-1 nor the behaviorally subeffective dose of U0126 altered ERK phosphorylation. Each bar represents the mean ± SEM percent change from vehicle (*$p < 0.05$, **$p < 0.01$). Insets are representative Western blots (n = 7-8/group).

object, $t_{(7)} = 0.86$, $p = 0.42$), suggesting that JNK activation is necessary for G-1 to enhance memory consolidation. In contrast, mice infused with G-1 + U0126 spent significantly more time than chance with the novel object ($t_{(8)} = 2.83$, $p = 0.02$) and moved object ($t_{(10)} = 2.48$, $p = 0.03$), suggesting that ERK activation is not necessary

for G-1 to enhance memory consolidation. We next examined the effects of JNK and ERK inhibition on G-1 mediated hippocampal cell signaling. Consistent with the behavioral data, ICV infusion of G-1 increased phosphorylation of both p46 JNK ($F_{(2,19)} = 6.56$, $p < 0.01$; Fig. 6A) and p54 JNK ($F_{(2,19)} = 6.47$, $p < 0.01$; Fig. 6B) 5 min after infusion. DH infusion of SP600125 abolished the effects of G-1 on p46 and p54 JNK (Fig. 6A,B). In contrast, G-1 and SP600125 did not significantly alter ERK phosphorylation (Fig. 6C). Unlike SP600125, U0126 did not block the GPER-mediated JNK activation (Fig. 7A,B). Whereas G-1 increased phosphorylation of both p46 JNK ($F_{(2,15)} = 4.44$, $p < 0.05$; Fig. 7A) and p54 JNK ($F_{(2,15)} = 6.68$, $p < 0.01$; Fig. 7B) 5 min after infusion, U0126 did not block the effects of G-1 on p46 JNK ($t_{(10)} = 2.35$, $p < 0.05$; Fig. 4H) and p54 JNK ($t_{(10)} = 2.34$, $p < 0.05$; Fig. 7B). Moreover, neither G-1 nor U0126 infusion altered ERK activation (Fig. 7C). These data suggest that ERK activation does not regulate G-1-induced hippocampal JNK activation. Together, these results support that activation of JNK, but not ERK, is essential for GPER to induce memory enhancement.

E_2-mediated hippocampal memory consolidation is independent of GPER and JNK activation

We have previously demonstrated that E_2 enhances hippocampal memory consolidation by ERα– or ERβ-mediated ERK activation in the DH (Fernandez et al., 2008; Boulware et al., 2013). In contrast, above data support that the G-1-induced enhancement of hippocampal memory consolidation is dependent on hippocampal JNK activation, rather than ERK activation. This finding begs the question of whether E_2-induced memory enhancements are also dependent on JNK and/or GPER activation. To address this issue, we first examined the effects of E_2 on JNK cell

signaling in the DH. Mice received bilateral DH infusion of the vehicle or E_2 (5 μg/ hemisphere), and the DH was dissected bilaterally 5 or 10 min later. DH E_2 infusion did not alter DH p46 JNK, and p54 JNK phosphorylation at either the 5 or 10 min time point (Fig. 8A,B), suggesting that E_2 does not activate JNK in the DH. As our previous studies (Fernandez et al., 2008; Boulware et al., 2013), DH E_2 infusion

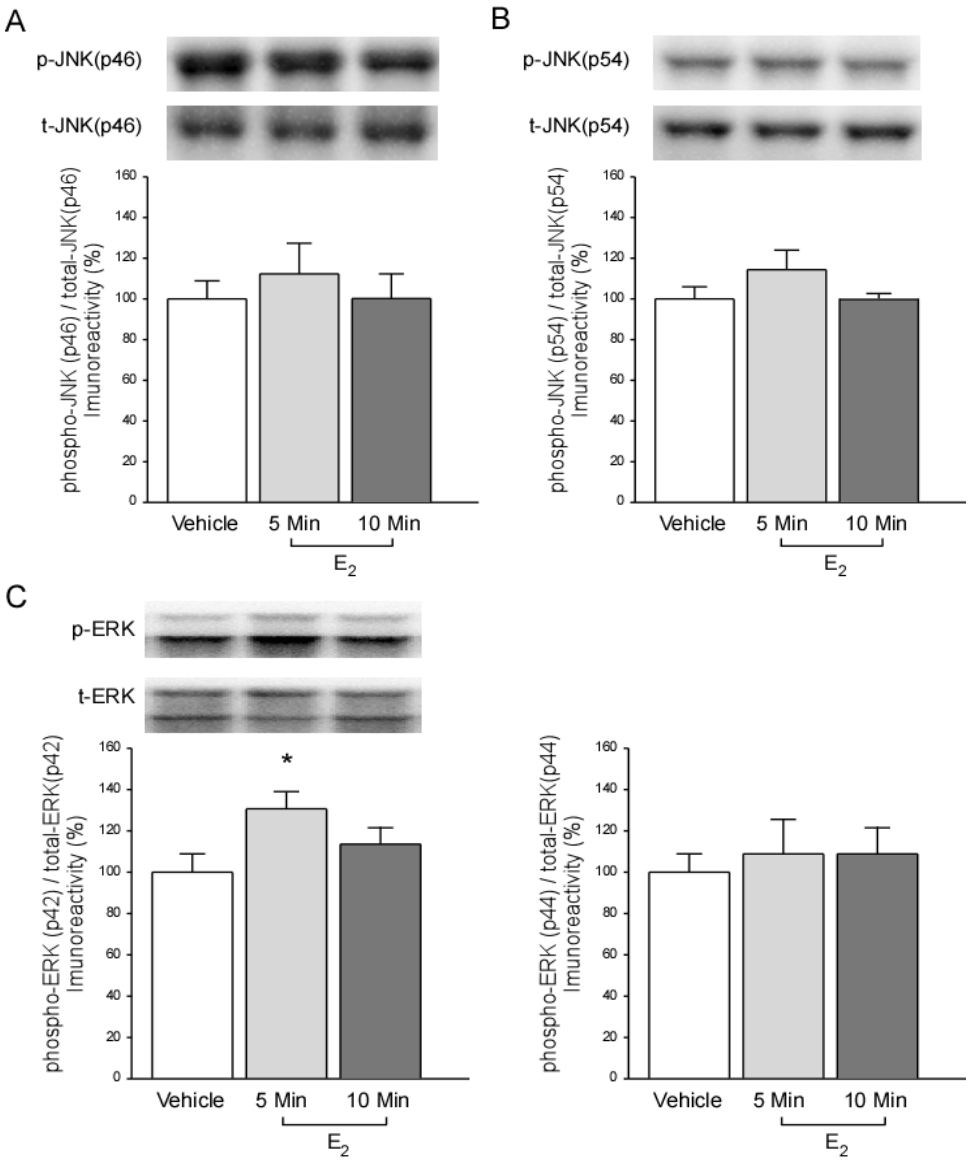

Figure 8. E_2 increases activation of ERK, but not JNK, in the DH. *A*, *B*, DH infusion of E_2 (5 μg/hemisphere) did not alter levels of phospho-p46 JNK (*A*) or phospho-p54 JNK (*B*) 5 or 10 min later. *C*, DH infusion of E_2 (5 μg/hemisphere) significantly increased phosphorylation of p42 ERK, but not p44 ERK, 5 min after infusion. Levels returned to baseline 10 min later. Each bar represents the mean ± SEM percent change from vehicle controls (*$p < 0.05$). Insets are representative Western blots. (n = 6/group).

increased phospho-p42-ERK 5 min after infusion ($F_{(2,15)} = 3.38$, $p < 0.05$; Fig. 8C) but had no effect on p44 ERK (Fig. 8C). These data suggest that E_2 increases activation of ERK, but not JNK, in the DH.

Next, we investigated the effects of GPER and JNK inhibition on E_2-mediated hippocampal cell signaling. Mice received ICV and DH infusions, respectively, of vehicle + vehicle, E_2 + vehicle, E_2 + SP600125, or E_2 + G-15, and DH tissue was collected 5 min later. As in our previous work (Boulware et al., 2013), ICV infusion of E_2 increased levels of phospho-p42 ERK ($F_{(3,20)} = 7.6$, $p < 0.01$), but not phospho-p44 ERK ($F_{(3,20)} = 0.7$, $p > 0.05$) (Fig. 9A). The increase in p42 ERK was not blocked by DH infusion of G-15 and SP600125 (G-15, $F_{(3,20)} = 7.6$, $p < 0.001$; SP600125, $F_{(3,20)} = 7.6$, $p < 0.05$; Fig. 9A). As with DH infusion, ICV infusion of E_2 did not alter phosphorylation of p46 JNK or p54 JNK, whether alone or in combination with DH infusion of G-15 and SP600125 (Fig. 9B,C). Together, these data reiterate that E_2 does not rapidly activate JNK in the DH and indicate that activation of JNK or GPER is not necessary for E_2 to activate ERK in the DH.

Given this finding, the next logical step was to determine whether JNK and GPER activation play a role in E_2-mediated hippocampal memory enhancement. To do so, we infused mice with vehicle, G-15 (1.85 ng/hemisphere) or SP600125 (2.75 ng/hemisphere) into the DH and vehicle or E_2 (10 μg) into the dorsal third ventricle immediately after OR and OP training. OR and OP retention were tested 48 and 24 hours later, respectively. In both tasks, mice receiving E_2 + vehicle showed a significant preference for the novel object ($t_{(5)} = 2.73$, $p = 0.04$; Fig 9D) and moved object ($t_{(7)} = 2.69$, $p = 0.03$; Fig 9E), in agreement with our previous work (Boulware et al., 2013). Consistent with the lack of JNK activation observed above, SP600125

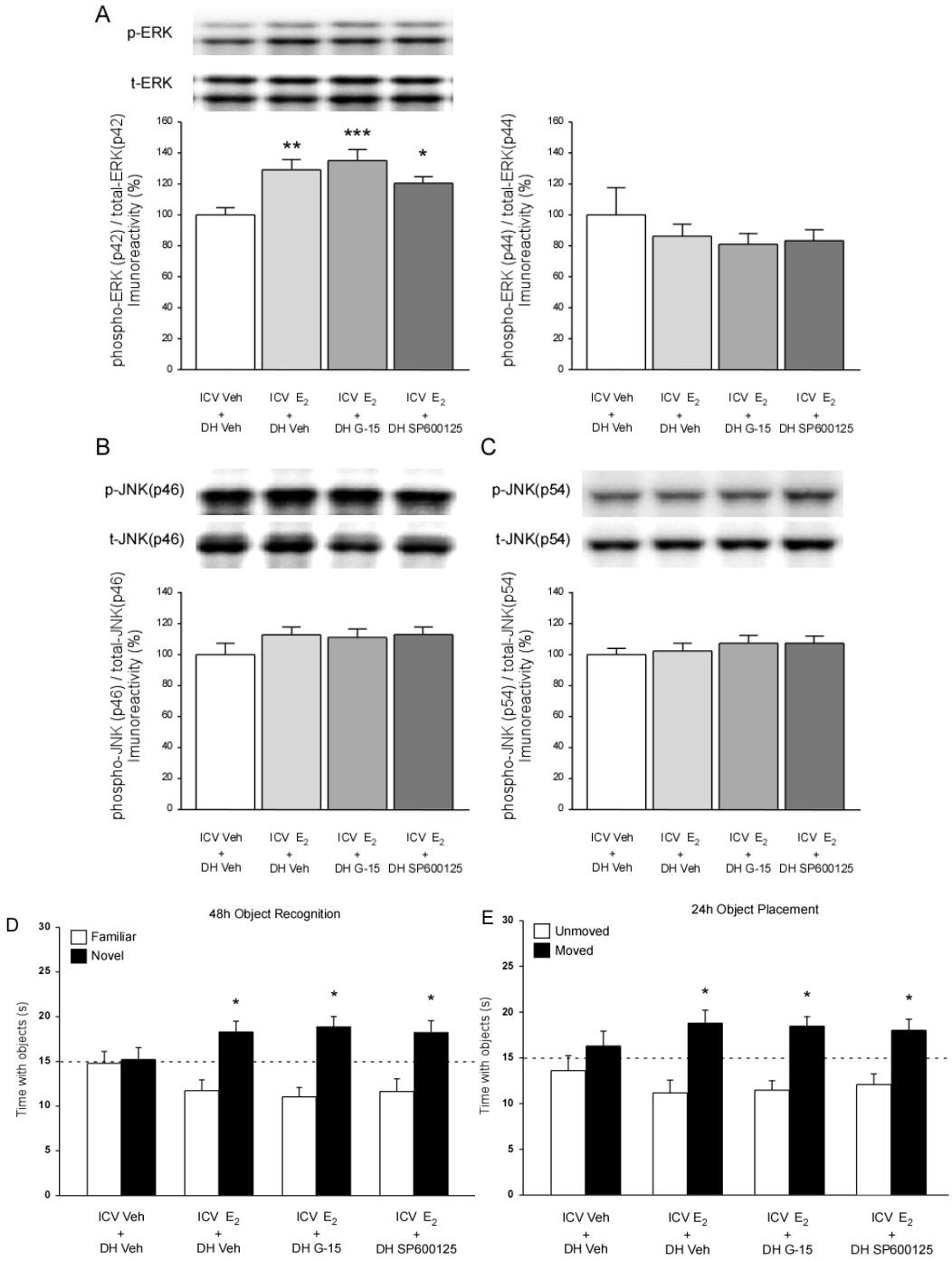

Figure 9. GPER and JNK inhibition do not affect the E_2-mediated memory enhancement and cell signaling in the DH. *A*, ICV infusion of E_2 (10 μg) increased phospho-p42 ERK levels 5 min after infusion; this effect was not blocked by DH infusion of G-15 or SP600125. ICV infusion of E_2 (10 μg) did not alter p44 ERK phosphorylation (n = 6/group). *B, C*, ICV infusion of E_2 did not alfter p46 JNK (*B*) or p54 JNK (*C*) phosphorylation 5 min after infusion when infused with vehicle, G-15, or SP600125 phosphorylation (n = 6/group). Each bar represents the mean ± SEM percent change from vehicle (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Insets are representative Western blots. *D, E*, Immediately after OR or OP training, mice received DH infusion of vehicle, G-15 (1.85 ng/hemisphere), or SP600125 (2.75 ng/hemisphere) followed by ICV infusion of vehicle or E_2 (10 μg). ICV infusion of E_2 significantly enhanced OR memory (*D*) and OP memory (*E*), and these effects were not blocked by G-15 or SP600125 (*D, E*), suggesting that neither GPER nor JNK activation is necessary for E_2 to enhance hippocampal memory consolidation (n = 6-12/group). Each bar represents the mean ± SEM time spent with the novel or moved object (*$p < 0.05$ relative to chance).

did not prevent E_2 from enhancing OR or OP memory consolidation (Fig. 9D,E), as mice receiving E_2 + SP600125 spent significantly more time with the novel object ($t_{(11)} = 2.36$, $p = 0.04$) and moved object ($t_{(9)} = 2.45$, $p = 0.04$). Interestingly, G-15 also did not block E_2-induced memory enhancements in either task (Fig. 9D,E), as demonstrated by the fact that mice receiving E_2 + G-15 spent significantly more time with the novel object ($t_{(5)} = 3.67$, $p = 0.01$) and moved object ($t_{(6)} = 3.36$, $p = 0.02$). These results suggest that neither JNK nor GPER activation in the DH is necessary for E_2 to enhance hippocampal memory consolidation.

Discussion

The present study provides the first evidence that GPER, a putative estrogen receptor, regulates hippocampal memory consolidation in young ovariectomized female mice in an E_2-independent manner. This conclusion is supported by several novel findings. First, GPER activation in the DH enhances OR and OP memory consolidation and increases JNK, but not ERK, phosphorylation in the DH. Second, the memory-enhancing effects of GPER activation are blocked by inhibition of JNK, but not ERK, in the DH. Finally, E_2 infusion increases ERK, but not JNK phosphorylation in the DH, and the memory-enhancing effects of E_2 are blocked by inhibition of ERK, but not JNK or GPER activation. Collectively, these data indicate that E_2 enhances hippocampal memory consolidation in females by activating ERK, whereas GPER enhances hippocampal memory consolidation by activating JNK. As such, the data suggest that GPER in the DH does not function as an estrogen receptor to facilitate memory consolidation.

Our findings showing that G-1 enhanced OR and OP memory consolidation are consistent with previous studies demonstrating that systemic injections of G-1 enhanced spatial learning and memory in ovariectomized rats (Hammond et al., 2009; Hammond and Gibbs, 2011; Hawley et al., 2014). However, the rat data do not permit definitive conclusions about the role of hippocampal GPER in memory because systemic treatments do not specifically affect the hippocampus. Therefore, we used dorsal hippocampal infusions of G-1 to pinpoint the role of hippocampal GPER in regulating memory consolidation. To ensure that the effects of G-1 were specific to GPER, we tested whether G-15 could antagonize the effects of G-1, as some studies have indicated that G-1 can act in a GPER-independent manner. For example, in breast cancer cell lines, G-1 has been found to interact with an ER-α variant, ER- α

36, but not with GPER (Kang et al., 2010). Additionally, G-1 suppressed the proliferation of ovarian and breast cancer cells, whereas GPER siRNA or G15 did not attenuate the effects of G-1, suggesting that G-1 can act in a GPER-independent manner in cancer cell lines (Wang et al., 2012). In contrast to these proliferative cells, we found that G-15 infusion into the DH prevented G-1 from enhancing OR and OP memory consolidation, as well as increasing JNK phosphorylation. These data suggest that the effects of G-1 on memory and JNK activation are mediated by GPER in the hippocampus. Interestingly, although higher doses of G-15 on their own impaired both OR and OP memory consolidation, OP appeared to be a bit more sensitive to G-15 than OR at the doses tested. Nevertheless, our finding that post-training DH infusion of G-15 dose-dependently impaired memory consolidation is consistent with previous data showing that chronic systemic treatment with G-15 dose-dependently impaired spatial working memory in ovariectomized rats (Hammond et al., 2012). Together, these data suggest an important role of GPER in hippocampal memory processes.

The role of ERK in GPER-mediated memory enhancement

The molecular mechanisms through which GPER influences hippocampal memory have not been investigated previously. Therefore, one of the primary goals of this thesis was to pinpoint possible downstream effectors of GPER activation in the mouse hippocampus. Based on our previous findings showing that p42 ERK activation is necessary for E_2 and agonists of ERα and ERβ to enhance OR and OP memory (Fernandez et al., 2008; Boulware et al., 2013), our initial hypothesis was that p42 ERK phosphorylation would also be necessary for G-1 to enhance memory. This hypothesis was also supported by other studies showing that activation of GPER

can activate the ERK pathway in pancreatic beta cells and the ERK activation effect is removed in GPER knockout mice model and in GPER depletion model by small interfering RNA (Maggiolini and Picard, 2010; Sharma and Prossnitz, 2011). We first showed that G-1 does not affect p42 or p44 ERK phosphorylation in the DH 5, 15, or 30 min after infusion. These time points were selected based on our previous studies demonstrating that DH E_2 infusion increases p42 ERK phosphorylation 5 min after DH infusion (Fernandez et al., 2008; Boulware et al., 2013; Fortress et al., 2013b). G-1 has a slightly slower effects on the rapid mobilization of intracellular calcium ($t_{1/2} \approx$ 30 s) than E_2 ($t_{1/2} \approx 2$ s) (Bologa et al., 2006), and it may take longer for G-1 to activate ERK than E_2. Therefore, we included the 15 and 30 min time points as well as 5 min time point. However, G-1 infusion did not alter ERK activation at any time point. This finding is consistent with data from vascular smooth muscle cells showing that E_2, but not GPER, increases ERK phosphorylation in these cells (Ortmann et al., 2011).

To further explore possible effects of G-1 on ERK signaling, we measured whether G-1 regulated activation of PI3K and Akt, based on our previous finding that phosphorylation of PI3K/Akt signaling is necessary for E_2 to activate ERK in the DH and enhance OR memory consolidation (Fan et al., 2010; Fortress et al., 2013b). Moreover, several studies show that GPER can regulate Akt cell signaling in numerous cell lines (Moriarty et al., 2006; Maggiolini and Picard, 2010) and in rats (Jang et al., 2013). As with ERK, however, we found that DH infusion of G-1 did not increase PI3K or Akt phosphorylation at any time point. Indeed, Akt phosphorylation was decreased 30 min after infusion, the reason for which is unclear. Nevertheless, the fact that G-1 did not increase PI3K and Akt phosphorylation in the DH as observed

after E_2 infusion indicates that G-1 does not activate multiple aspects of ERK signaling in the female mouse DH.

These data led us to hypothesize that ERK activation would not play a role in the ability of G-1 to enhance OR and OP memory consolidation. Consistent with this hypothesis, we found that the ERK inhibitor U0126 did not prevent G-1 from enhancing OR or OP memory consolidation. These results demonstrate for the first time that ERK activation is not necessary for GPER to enhance hippocampal memory consolidation in female mice. Although this finding is novel as it relates to memory, it is consistent with reports from peripheral tissues showing that the ERK inhibitors U0126 and PD98059 do not prevent G-1 from inducing endothelium-dependent vasorelaxation in rat aorta (Jang et al., 2013) or DNA synthesis in human epithelial cells (Holm et al., 2011). These few examples do not permit any general conclusions about the role of ERK in mediating the cellular effects of GPER activation, the present data provide evidence that ERK is not involved GPER-mediated memory regulation.

The role of JNK in GPER-mediated memory enhancement

Given the unexpected lack of a role for ERK in GPER-induced memory enhancement, we next sought to identify other signaling pathways through which GPER may mediate memory consolidation. We focused on JNK signaling, since this MAPK is activated by various G proteins (Goldsmith and Dhanasekaran, 2007) and is involved in regulating synaptic plasticity (Tararuk et al., 2006; Waetzig et al., 2006; Kim et al., 2007). We demonstrated that GPER activation led to rapid phosphorylation of both JNK isoforms in the DH, an effect that was blocked by DH infusino of the JNK inhibitor SP600125, but not the ERK inhibitor U0126. In addition, we found that

G-1 increased phosphorylation of the downstream JNK transcription factor ATF2, suggesting that the G-1 induced phosphorylation of JNK also activated nuclear transcription. Importantly, we found that activation of JNK, but not ERK, in the DH is necessary for GPER to faciliate memory consolidaion in both the OR and OP tasks. As such, these data demonstrate that JNK activation, but not ERK activation, is necessary for GPER enhance hippocampal memory consolidation.

Although JNK has been studied in the context of cellular stress and apoptosis (Kyriakis and Avruch, 2001; Reinecke et al., 2013), JNK has also been shown to play an important role in synaptic plasticity, neuronal regeneration, and development in the central nervous system (Tararuk et al., 2006; Waetzig et al., 2006). However, its role in learning and memory has been understudied, and existing data provide conflicting results. For example, some studies suggest an important role of JNK activation in long-term inhibitory avoidance memory and in short-term synaptic plasticity and long-term depression (Bevilaqua et al., 2007; Li et al., 2007; Carboni et al., 2008). However, other data indicate that JNK negatively regulates short-term memory in the hippocampus (Bevilaqua et al., 2003). Duration of JNK activation may play an important role in the resulting effects on memory, as suggested by data showing that short-term JNK activation facilitates hippocampal memory and synaptic plasticity, whereas prolonged JNK activation leads to memory deficits and neurodegeneration (Sherrin et al., 2011). Although our findings cannot directly speak to the inconsitencies in the JNK literature, our findings provide much needed additional information on the role of JNK in hippocampal memory. These data suggest that JNK is an essential mediator of GPER-induced memory modulation. However, as will be discussed below, JNK appears to play no role in E_2-induced memory modulation.

The estrogen receptor that doesn't act like an estrogen receptor

Evidence that GPER is an estrogen receptor comes from data showing that E_2 binds GPER with high affinity (Revankar et al., 2005; Moriarty et al., 2006; Prossnitz et al., 2007). However, other studies suggest that GPER acts independently of E_2. For example, a study using endothelial cells from ERα/ERβ-deficient mice demonstrated that E_2 could not activate cAMP or ERK pathways, despite the presence of GPER (Pedram et al., 2006) and cells, COS-7 and CHO (Chinese hamster ovary) cells, transfected with GPER failed to signal in response to E_2 (Otto et al., 2008). Another study revealed that treatment with G-15 or downregulation of GPER expression with GPER shRNA did not prevent E_2-mediated apoptosis in rat aortic vascular endothelial cells (Ding et al., 2014). Further, rapid extranuclear E_2 signaling in breast cancer cells involved only ERα and ERβ but not GPER (Madak-Erdogan et al., 2008), and the neuroprotective effects of E_2 on post ischemic injury are not dependent on GPER (Lamprecht and Morrison, 2014). Moreover, some investigators insist that GPER is not a true ER, but potentially has a collaborative role in mediating the biological actions of estrogens (Levin, 2009). Such studies have stimulated extensive debate about whether GPER acts as a true estrogen receptor (Langer et al., 2010).

The present study adds to the debate by showing that GPER and E_2 do not enhance memory via the same cell signaling mechanisms. As we have previously shown, E_2 and agonists of ERα and ERβ require ERK activation in the DH to enhance OR and OP memory consolidation in ovariectomized female mice (Fernandez et al., 2008; Boulware et al., 2013; Fortress et al., 2013b). However, the present study found no role of ERK in the memory-enhancing effects of GPER. Furthermore, the present study found that E_2 did not phosphorylate either isoform of JNK at any time point

examined, nor did the JNK inhibitor SP600125 prevent E_2 from enhancing OR or OP memory consolidation. These data demonstrate not only that E_2 does not activate JNK in the DH, but also that JNK activation is not necessary for E_2 to enhance hippocampal memory consolidation. Although these data provide strong support that GPER and E_2 independently regulate memory formation, more definitive evidence comes from the fact that G-15 does not prevent E_2 from enhancing either OR or OP memory consolidation. These data demonstrate that GPER activation is not necessary for E_2 to enhance hippocampal memory consolidation, and suggest that GPER does not function as an estrogen receptor in the dorsal hippocampus. Although we cannot presently rule out potential interactions between GPER and ERα or ERβ, we find this possibility unlikely given how closely ER agonists mimic the effects of E_2 on memory and ERK signaling (Boulware et al., 2013).

Further studies and conclusion

The surprising finding that GPER does not act as an estrogen receptor in the dorsal hippocampus begs the question of whether GPER directly interacts with ERα or ERβ. On the basis of our data, we could first hypothesize that no such interactions take place and that GPER is not an estrogen receptor, at least in the hippocampus. If GPER does not work as estrogen receptor, the alternative natural ligand for GPER might be aldosterone. Some studies have indicated that the potential role of aldosterone in GPER activation in vascular smooth muscle cells (Brailoiu et al., 2013; Gros et al., 2013), still it needs further investigation (Filardo and Thomas, 2012). Although several studies assert that GPER acts independently of E_2 (Pedram et al., 2006; Otto et al., 2008; Ding et al., 2014), it is difficult to ignore the many other

studies showing that E_2 activates GPER (Revankar et al., 2005; Thomas et al., 2005; Moriarty et al., 2006; Prossnitz et al., 2007; Langer et al., 2010). Therefore, an alternative hypothesis is that the activation of intracellular ERs may inhibit GPER activation. Thus, activation of either ERα or ERβ might be able to suppress GPER activation because E_2 has higher binding affinity on ERα and ERβ than GPER; competitive radiometric binding assay showed the K_d value of E_2 on ERα (0.30 nM) and ERβ (0.90 nM) in human endometrial cancer (HEC-1) cells, and the K_d value of E_2 on GPER (3.0 nM) in human embryonic kidney (HEK)293 cells (Sun et al., 1999; Thomas et al., 2005). To examine potential interactions between intracellular ERs and GPER, we may be able to test whether ERα and ERβ antagonists block the effects of G-1 on memory and JNK activation. We can also examine physical interactions among the receptors using sucrose fractionation and co-immunoprecipitation (CoIP) as we have described previously(Boulware et al., 2013) . Delineating such interactions would provide important insight into how the intracellular ERs and GPER may interact to regulate hippocampal memory.

Future studies could also better elucidate the role of JNK signaling in hippocampal formation, based on our findings that GPER activation enhances hippocampal memory via the JNK signaling pathway. Given how little is known about the role of JNK in memory, one possible future direction would be to determine the molecular mechanisms through which JNK affects hippocampal memory. Two avenues of research may be particularly promising: 1) determining how JNK regulates PSD-95 protein, and 2) identifying how JNK modulates gene expression. Because interactions between GPER and PSD-95 have been identified in hippocampal dendritic spines (Akama et al., 2013) and JNK kinase activity is involved in the

regulation of the PSD-95 to recruit synaptic AMPA receptors (Kim et al., 2007), the JNK-PSD-95 relationship is worthy of further study to elucidate how JNK affects hippocampal memory. Physical interaction between JNK and PSD-95 can be examined by Co-IP and JNK inhibition effect on PSD-95 expression can be tested by western blot or PCR. JNK-mediated gene expression in the hippocampus would also be interesting to examine because we showed that G-1 activated the JNK downstream transcription factor, ATF-2. ATF-2 works as a transcription factor responding to nerve growth factor in sympathetic neurons (Lau and Ronai, 2012), therefore, microarray technique will provide potential target gene for ATF-2 in the hippocampus and the gene expression will be confirmed by RT-PCR. If we find JNK-mediated changes in gene expression, then it would be interesting to examine the epigenetic processes that might regulate this expression. For example, our laboratory has demonstrated that histone acetylation and DNA methylation are necessary for E_2 to enhance OR memory consolidation in ovariectomized female mice (Zhao et al., 2010; Zhao et al., 2012). In addition, E_2-induced histone H3 acetylation was dependent on ERK activation (Zhao et al., 2010; Zhao et al., 2012), indicating that changes in histone acetylation are triggered by cell signaling mechanisms. To our knowledge, JNK-induced regulation of epigenetic processes has not been investigated, and so is an area ripe for investigation.

In conclusion, the present study provides the first evidence that GPER activation can enhance hippocampal memory consolidation in JNK dependent manner and that E_2-mediated memory enhancement is independent of GPER and JNK activation. These results do not support a role for GPER in the memory-enhancing effects of E_2, although GPER activation has similar memory-enhancing effects as E_2. This interesting finding may have important implications for the future design of

estrogen-based therapies for reducing the risk of age-related memory decline and Alzheimer's disease in women (Yaffe et al., 1998; Zandi et al., 2002). Therefore, better understanding of GPER function could provide important opportunities for the development of new therapies that would provide the cognitive benefits of estrogen without potentially dangerous side effects.

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