Dickkopf-1 Blocks $17\beta$-estradiol-enhanced Object Memory Consolidation in Ovariectomized Female Mice

Lisa Taxier

University of Wisconsin-Milwaukee

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DICKKOPF-1 BLOCKS 17β-ESTRADIOL-ENHANCED OBJECT MEMORY

CONSOLIDATION IN OVARIECTOMIZED FEMALE MICE

by

Lisa Taxier

A Thesis Submitted in
Partial Fulfillment of the
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ABSTRACT

DICKKOPF-1 BLOCKS 17β-ESTRADIOL-ENHANCED OBJECT MEMORY CONSOLIDATION IN OVARIECTOMIZED FEMALE MICE

by

Lisa Taxier

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

The memory-enhancing effects of 17β-estradiol (E2) are dependent upon rapid activation of several cell signaling cascades within the dorsal hippocampus (DH). Among many cell-signaling pathways that mediate memory processes, Wnt/β-catenin signaling has emerged as a potential key player. However, whether E2 interacts with Wnt/β-catenin signaling to promote memory consolidation is unknown. Therefore, the present study examined whether Wnt/β-catenin signaling within the DH is necessary for E2-induced memory consolidation in both object recognition and object placement tasks. Here, ovariectomized mice received immediate post-training infusions of E2 or vehicle into the dorsal third ventricle plus the endogenous Wnt/β-catenin antagonist Dickkopf-1 (Dkk-1) or vehicle into the dorsal hippocampus to assess whether the memory-enhancing effects of E2 depend on activation of Wnt/β-catenin signaling. Our results suggest that Dkk-1 blocks E2-induced memory enhancement as hypothesized, but may operate in an unexpected manner by only moderately blunting Wnt/β-catenin signaling while concurrently activating Wnt/JNK signaling. The current study provides novel insight into the mechanisms by which E2 enhances memory consolidation in the DH, as well as critical information about the mechanistic actions of Dkk-1.
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Introduction

The sex steroid hormone 17β-estradiol (E₂) is a robust positive mediator of hippocampal plasticity and memory (Fernandez et al; 2008, Fortress et al, 2014; McClure et al, 2013; Woolley et al, 1997; Zhao et al, 2010). However, the downstream mechanisms of E₂-enhanced memory processes still remain to be fully characterized. Our laboratory and others have demonstrated that infusion of E₂ into the female mouse dorsal hippocampus (DH) rapidly activates several cell-signaling cascades, including the extracellular signal-regulated kinase (ERK), mammalian target of rapamycin (mTOR)-mediated protein synthesis, and phosphatidylinositol 3-kinase (PI3K) pathways (Fernandez et al, 2008; Fan et al, 2010; Fortress et al, 2013b). Moreover, this activation is necessary for E₂ to facilitate object recognition and spatial memory consolidation in ovariectomized (OVXed) female mice (Boulware et al., 2013; Fernandez et al, 2008; Fan et al, 2010, Kim et al, 2016), illustrating that rapid activation of DH cell signaling plays an essential role in the memory-enhancing effects of E₂. One cell signaling cascade that has been implicated in memory processes is the Wnt/β-catenin, or canonical Wnt signaling pathway. Our laboratory demonstrated that rapid activation of the Wnt/β-Catenin signaling pathway in the DH is critical for object recognition memory consolidation in male mice, adding to a growing body of literature implicating this pathway as an important mediator of memory processes (Fortress et al, 2013). However, estrogenic mediation of the Wnt/β-catenin signaling pathway has yet to be examined in vivo as an important mediator of memory consolidation. The aim of the present experiment was to examine whether the memory-enhancing effects of E₂ are dependent upon Wnt/β-catenin signaling pathway activation.
Wnt signal transduction: an overview

The name “Wnt” comes from combining the gene wingless, identified in Drosophila and important for cell polarity, with the homologous gene in vertebrates, integrated or int-1 (Nusse and Varmus, 1982). The first identified Wnt molecule, Wnt1, was identified as a mediator of mammary tumor development, and several other Wnt proteins were initially studied in the context of both cancer pathology and embryonic development (Nusse and Varmus, 1982). There are 19 identified discrete Wnt proteins, which act as extracellular glycoproteins that bind to the seven transmembrane domain Frizzled (Fz) receptors to initiate downstream signaling. Within the secretory pathway, Wnts are translated and targeted to the endoplasmic reticulum, where they are eventually secreted in vesicles and trafficked to the extracellular space. Although not all of the precise mechanisms of secretion, binding, and even manufacturing of specific Wnt proteins have been elucidated, multiple researchers have demonstrated that two primary posttranslational modifications, acylation and N-glycosylation, are important for Wnt protein function (Tang et al, 2012). Almost all Wnts are acylated by the acyltransferase Porcupine, which appends palmitoleate while Wnts are in the endoplasmic reticulum (Langton et al, 2016). Furthermore, lipidation of Wnts appears to be a critical modification that allows for interaction with the Fz receptor (Langton et al, 2016). However, the ways in which these modifications and others may contribute to Wnt action, and in which contexts, have yet to be fully characterized. For example, lipidation, while critical for Wnt action on cell targets, may affect how far Wnts may travel in the extracellular space, leading many researchers to posit that Wnts act primarily in short-range signaling (Willert et al, 2003; Niehrs, 2010; Farin et al, 2016). Conversely, others have suggested that Wnts may be able to bind to other molecules in the extracellular space that may affect solubility and ability to exert longer-range signaling effects (Langton et al, 2016). More work
remains in order to fully describe the process of Wnt secretion, beginning from the level of synthesis and extending to characterizing how Wnts function in discrete environments.

Since the initial discovery of Wnt molecules in both *Drosophila* and vertebrates, an expanding spectrum of roles for Wnt signaling has emerged. Our current knowledge of Wnts in and outside of the nervous system includes roles for Wnt signaling in cell fate determination, motility, and as it relates to the current work, memory processes (Komiya and Habas, 2008; Fortress and Frick, 2015). Indeed, Wnt signaling is critical for normal hippocampal development, and later on plays a key role in mediating synaptic plasticity, long-term potentiation, and dendritic morphogenesis within the hippocampus (Lee et al, 2000; Chen et al, 2006; Dickins and Salinas, 2013; Rosso et al, 2005). Although there are many signaling pathways involving Wnt molecules, Wnt signaling is traditionally classified by three distinct pathways: the Wnt/planar cell polarity (PCP) pathway, the Wnt/Calcium pathway, and the canonical or Wnt/β-catenin signaling pathway.

**Wnt/β-Catenin signaling**

The most well known and well characterized Wnt signaling pathway is the Wnt/β-catenin signaling pathway (Fig 1A). The functional result of this pathway, as its name suggests, is the translocation of β-catenin into the cell nucleus, where it acts as a co-factor for existing nuclear transcription factors, and facilitates gene transcription. When Wnt/β-catenin signaling is active, Wnt ligands bind to Frizzled (Fz) receptors and low-density lipoprotein receptor-related protein 5 (LRP5) or LRP6 receptors, leading to the phosphorylation of glycogen synthase kinase-3 β (GSK3β), which prevents the phosphorylation of β-catenin and allows it to accumulate and translocate into the nucleus (Ciani and Salinas, 2005). In the nucleus, β-catenin binds to the transcription factor TCF/LEF, which then increases expression of target genes including
Cyclin D1 and c-myc. In the absence of Wnt ligand binding to Fz or LRP5/6, GSK3β phosphorylates β-catenin, allowing for ubiquitination and targeting β-catenin for proteosomal degradation. Wnt/β-catenin signaling can also be inhibited by Dickkopf-1 (Dkk-1), an endogenous protein that prevents Wnt ligands from complexing with Fz and LRP5/6 co-
receptors (Bafico et al, 2001). The resulting unphosphorylated GSK3β then phosphorylates β-catenin, which is then degraded in the proteasome (Inestrosa & Arenas, 2010) so that it cannot initiate gene transcription. Importantly, although Dkk-1 is traditionally thought of as a specific antagonist of the Wnt/β-catenin signaling pathway, emergent evidence suggests a potential role for Dkk-1 in altering signaling in non-canonical Wnt pathways. In fact, multiple researchers have suggested that Dkk-1 binding to LRP5/6 may shift the balance away from Wnt/β-catenin signaling by allowing other non-canonical Wnts to bind to the Frizzled receptor at the membrane, effectively shifting the balance towards β-catenin-independent signaling (Endo et al, 2008; Caneparo et al, 2007).

**β-Catenin-independent Wnt signaling pathways**

Although Wnt/β-catenin signaling was the primary pathway of interest in the present study, additional Wnt signaling pathways may also play an as of yet undescribed role in the memory-enhancing effects of E2, and thus merit discussion. Furthermore, given evidence that Dkk-1 may interact with β-Catenin-independent pathways, a description of key players in β-Catenin-independent signaling is warranted. Here we will focus on describing the two other primary Wnt signaling pathways, Wnt/PCP signaling and Wnt/Calcium signaling.

The Wnt/PCP signaling pathway, or as I will refer to it subsequently, the Wnt/JNK pathway, plays a key role in regulating cytoskeletal dynamics and cell polarity. This pathway was initially described as a critical signaling event that allowed for planar, organized cellular activity (thus the term planar cell polarity), such as orientation of fly wing hairs or sensory hair cells in the mammalian inner ear (Boutros et al, 1998; Shulman et al, 1998). In mammalian systems, Wnt/JNK signaling is largely homologous to Wnt/PCP signaling. In this pathway, instead of complexing with LRP5/6, the Fz receptor instead complexes with co-receptors such as
RYK or MUSK (Nierhs, 2012). The Fz/co-receptor complex then activated Dvl, which signals to monomeric GTPases Rho and Rac. Rho then stimulates ROCK, while Rac activates JNK to ultimately regulate downstream target genes involved in cytoskeletal dynamics (Olivia et al., 2013; Rosso and Inestrosa, 2013; Fig 1B). Importantly, although Dkk-1 is thought to be a Wnt/β-catenin signaling antagonist, recent evidence suggests that Wnt/JNK signaling may also be targeted by Dkk-1. For example, one recent study describes synapse disassembly mediated by Dkk-1 activation of Wnt/JNK signaling (Marzo et al., 2016). Although the mechanism through which Dkk-1 interacts with Wnt/JNK signaling is unclear, it may be the case that Dkk-1 shifts the balance away from Wnt/β-catenin signaling activity towards Wnt/JNK signaling activity.

The third primary Wnt signaling pathway, and the second primary non-canonical Wnt signaling pathway, is the Wnt/Calcium signaling pathway (Fig 1B). Here, as its name suggests, the net result of signaling activation is an increase in intracellular calcium activity, and a resulting change in transcriptional activity through transcription factors such as CREB. As in both Wnt/β-catenin signaling and Wnt/JNK signaling, Wnts bind to membrane Fz receptors and initiate a signal cascade through Dvl (Slularski et al., 1997). When this signaling pathway is “on,” phospholipase C is activated, resulting in increased intracellular Ca^{2+}, which allows for subsequent increases in diacylglycerol, calcineurin and Ca^{2+}/calmodulin dependent protein kinase II (CaMKII). Protein kinase C is also activated, which potentiates intracellular Ca^{2+} increases (Niehrs 2012, Hogan et al 2003). The primary function of this pathway is thought to be related to axon guidance, although several researchers have also demonstrated a role for this pathway in regulating synaptic plasticity (McQuate et al., 2017; Wayman et al., 2006).
**Wnt/β-Catenin signaling and memory processes**

Increasing evidence suggests that the Wnt/β-catenin signaling pathway plays an important role in learning and memory processes, especially within the hippocampus. For instance, spatial learning in the Morris water maze increases levels of the canonical Wnt proteins Wnt7a/b in area CA3 of the hippocampus in male rats (Tabatadze et al., 2012). In addition, contextual fear conditioning increases expression of Wnt3a, whereas intrahippocampal infusion of a Wnt3a antibody impairs both the acquisition and consolidation phases of contextual fear conditioning, but not the expression phase (Xu et al., 2015). Wnt7a/b is increased in hippocampal cell bodies and is subsequently trafficked to dendrites after glutamate activation, suggesting a role for canonical Wnts in regulating synaptic plasticity (Tabatadze et al., 2014). Notably, expression of the Wnt antagonist Dickkopf-1 (Dkk-1) within the brain increases with age in both rodents and humans. However, knockout of Dkk-1 in the aging mouse hippocampus increases neurogenesis and facilitates spatial memory consolidation (Seib et al., 2013). These data implicate a role for dysfunctional Wnt/β-catenin signaling in cognitive aging. Our laboratory has shown that blockade of Wnt/β-catenin signaling with Dkk-1 prevents memory consolidation in an object recognition task in male mice (Fortress et al., 2013). Conversely, activation of Wnt signaling *in vivo* with Wnt-activating small molecule (WASP-1) enhances episodic memory on a memory flexibility task in the water maze, and concurrently increases basal excitatory synaptic transmission and facilitates LTP in adult mice (Vargas et al., 2015). Combined, these data demonstrate the involvement of Wnt/β-catenin signaling in memory processes.

**E₂, cell signaling, and memory processes**

Our laboratory has demonstrated repeatedly that DH infusion of E₂ in OVXed female mice activates multiple cell-signaling pathways within 5 min in the DH that regulate learning and
memory processes, such as the extracellular signal-regulated kinase (ERK) pathway, mammalian target of rapamycin (mTOR)-mediated protein synthesis, and phosphatidylinositol 3-kinase (PI3K) cascades (Fernandez et al, 2008; Fan et al, 2010; Fortress et al, 2013b). Moreover, this activation is necessary for E2 to facilitate object recognition and spatial memory consolidation in OVXed female mice (Boulware et al., 2013; Fernandez et al, 2008; Fan et al, 2010, Kim et al, 2016), illustrating that rapid activation of DH cell signaling plays an essential role in the memory-enhancing effects of E2.

The above-described rapid effects of E2 on cell-signaling activation in the DH occur within seconds to minutes, and are mediated by E2 binding to membrane-bound estrogen receptors (ERs) or ERs in close proximity to the cell membrane. Conversely, non-classical effects, which occur within hours or days, are mediated by a complex containing E2 and an estrogen receptor (ER) binding to an estrogen response element on DNA, which leads to changes in gene expression. Among the most well-characterized estrogen receptors are ERα, ERβ, and G protein-coupled estrogen receptor (GPER). Intracellular receptors ERα and ERβ are known to mediate classical effects of E2 within the DH, whereas GPER, which is present within cell membranes, is less well characterized. However, recent data from our lab suggest that activation of hippocampal GPERs enhances OR and OP memory in ovariectomized females (Kim et al, 2016). Interestingly, GPER-mediated memory enhancement appears to occur independently of E2, and instead depends upon JNK activation.

**Wnt/β-Catenin signaling and E2 interactions**

Although our laboratory has demonstrated the critical importance of Wnt/β-catenin signaling for object recognition in male mice, the putative role of Wnt/β-Catenin signaling in mediating the mnemonic effects of E2 is unknown. However, evidence from multiple studies
suggests that E2 may activate Wnt/β-catenin signaling. For instance, E2 serves a neuroprotective function in animal models of cerebral ischemia by downregulating Dkk-1, which is usually present in abnormally high amounts in ischemia models, in area CA1 of the mouse hippocampus (Zhang et al, 2008). Furthermore, E2 increases levels of Wnt3a, a canonical Wnt ligand, in area CA1 of the hippocampus following experimental induction of cerebral ischemia in mice (Zhang et al, 2008). Estrogen receptor α (ERα) also complexes with GSK3β and β-catenin in the hippocampus. In the presence of E2, β-catenin dissociates from this complex, presumably allowing it to act downstream to increase transcription of Wnt/β-catenin target genes (Cardona-Gomez, 2003). Further, Cardona-Gomez et al (2004) demonstrate that ovariectomized female rats treated with E2 exhibit a transient increase in phosphorylated hippocampal GSK3β, further cementing a role for E2 in activating Wnt/β-catenin signaling. Another study suggests a role for E2 in mediating the interaction between the estrogen receptor coregulator protein Proline-, glutamic acid-, and leucine-rich protein 1 (PELP1) and the phosphorylation of GSK3β (Sareddy et al, 2015). Interestingly, the E2-induced increase in phosphorylation of GSK3β does not occur in PELP1 knockout mice, suggesting that PELP1 is critical for the E2-mediated increase in GSK3β phosphorylation (Sareddy et al, 2015). Together, these data suggest a potential role for E2 in mediating Wnt/β-catenin signaling. However, evidence describing the extent to which E2 activates this pathway to promote memory consolidation is lacking.

Therefore, the present study investigated whether Wnt/β-catenin signaling is necessary for the memory-enhancing effects of E2 previously observed in OVXed mice (Fernandez et al., 2008; Fortress et al., 2013; Boulware et al., 2013; Kim et al., 2016). Mice received bilateral DH infusion of Dkk-1 immediately after training in object recognition and object placement tasks, concurrent with intracerebroventricular (ICV) infusion of E2. Our findings suggest that Dkk-1
prevents E₂ from enhancing object recognition and spatial memory consolidation in OVXed mice via modest blockade of Wnt/β-catenin signaling and concurrent activation of Wnt/JNK signaling.

Methods

Subjects

Female C57BL/6 mice aged 8-10 weeks (Charles River, Wilmington, MA) were used for all studies. Mice were housed in groups of up to 5/cage until 24 h prior to surgery, at which time they were singly housed. Mice were maintained on a 12 h light/dark cycle with ad libitum access to food and water. All experimental protocols and procedures were approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee and are in accordance with National Institutes of Health guidelines or Guide for the Care and use of Laboratory Animals.

General experimental design

Three days after arrival in the lab, mice were ovariectomized and implanted with guide cannulae aimed at the dorsal hippocampus and dorsal third ventricle. Following a recovery period of one week, mice underwent behavioral training and testing in an object recognition (OR) task to measure object recognition memory and an object placement (OP) task to measure spatial memory. Unique sets of objects were used for each task. The order of testing in each task was counterbalanced across groups and separated by two weeks to allow the hippocampus to recover from infusion. Two weeks following completion of behavioral testing, mice were re-infused and dorsal hippocampi were collected at various time points following infusion for Western blotting experiments.
Surgery

All surgeries were conducted starting 3 days after arrival in the laboratory as described previously (Boulware et al., 2013; Fortress et al., 2013). Mice were anesthetized with isoflurane (5% for induction, 2% for maintenance) in 100% oxygen and placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA) from which they underwent ovariectomy and cannula implantation in the same surgical session as described previously (Boulware et al., 2013; Fortress et al., 2013). For initial experiments using single drugs (e.g. Dkk-1), mice were implanted with double guide cannulae (Plastics One, Roanoke, VA) aimed bilaterally at the dorsal hippocampus (DH; C232GC, 22 gauge; −1.7 mm AP, ±1.5 mm ML, and −2.3 mm DV). For experiments using concurrent infusion of E2 and Dkk-1, mice were implanted with three stainless steel guide cannulae aimed at the dorsal third ventricle (ICV; C232GC, 22 gauge; −0.9 mm AP, ± 0.0 mm ML, and −2.8 mm DV) and both sides of the DH as described above. Cannulae were fixed to the skull with dental cement (Darby Dental Supply, New York, NY) that served to close the wound. Dummy cannulae (C232DC; Plastics One) were inserted into each cannula to prevent clogging of the cannula tracts. Mice were given at least one week prior to the start of behavioral testing.

Drugs and infusions

The Wnt/β-catenin inhibitor Dickkopf-1 (Dkk-1) was dissolved in sterile 0.9% saline to a concentration of 5 ng/µl. A volume of 0.5 µl Dkk-1 or saline vehicle was infused bilaterally into each side of the DH at a rate of 0.5 µl/min for 1 min per hemisphere immediately following training in OR and OP. Cyclodextrin-encapsulated E2 (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile 0.9% saline to a concentration of 10 µg/µl. The vehicle consisted of 2-hydroxypropyl-β-cyclodextrin (HBC; Sigma-Aldrich, St. Louis, MO) dissolved in saline to the same concentration of cyclodextrin present in the cyclodextrin-encapsulated E2 solution. A total volume of 1 µl E2 or HBC was infused into the dorsal third ventricle at a rate of 0.5 µl/min for 2
min to allow for the same total volume as DH infusions at the same rate. This triple infusion protocol is routinely used by our lab to prevent potential damage to the DH from two infusions into the DH in rapid succession (Fernandez et al., 2008; Fan et al., 2010; Zhao et al., 2010, 2012; Boulware et al., 2013; Fortress et al., 2013). Here, we used triple cannulae to administer \( E_2 \) into the dorsal third ventricle adjacent to the DH while infusing Dkk-1 bilaterally into the DH. This protocol avoids potential damage to the DH from infusions of both compounds, and precludes possible interactions between the drugs within the DH and reduced drug efficacy that could result from the dilution of each drug in the larger ICV infusion volume.

**Behavioral testing**

Object recognition (OR) and object placement (OP) were used to measure object recognition and spatial memory as we have previously described (Fernandez et al., 2008; Fortress et al., 2013; Boulware et al., 2013; Kim et al., 2016; Tuscher et al., 2016). Mice were handled for 30 sec/day for three days prior to habituation. On the second day of handling, a Lego was placed in each home cage to habituate mice to objects during remaining days of handling and habituation. After three days of handling, mice were habituated to the empty testing apparatus (60 cm x 60 cm x 47 cm) for 5 min/day for two consecutive days. The next day, mice were habituated to the empty arena for 2 min prior to training with objects, removed from the arena and placed in a holding cage, and then placed back into the arena with two identical objects placed 5 cm from the upper left and right corners of the arena. Mice were then allowed to explore the objects until they had accumulated 30 sec of exploration time or until 20 min had elapsed. Immediately following training, mice received DH or DH+ICV drug infusions and were returned to their home cage. Conducting infusions immediately post-training allowed us to establish the effects of \( E_2 \) and/or Dkk-1 specifically on memory consolidation in the absence of
confounding effects on non-mnemonic performance factors (e.g., motivation, sensorimotor abilities) (Gresack and Frick, 2006; Fernandez et al., 2008). During OR testing, mice were returned to the arena and allowed to accumulate 30 s exploring one object that was identical to those used during training and a novel object. The location of the objects remained the same as during training. Mice that remember the identity of the training objects will spend more time than chance (15 s) exploring the novel object during testing, whereas mice that do not will not explore the novel object more than chance (Frick and Gresack, 2003). During OP testing, mice were allowed to explore objects identical to those used in training, however, one object was moved to a lower corner of the arena. Mice that remember the location of the training objects will spend more time than chance (15 s) exploring the moved object during testing (Boulware et al., 2013; Kim et al., 2016). For all experiments, time spent with the objects was recorded using ANYmaze software (Stoelting).

In our first experiment, testing was conducted 24 h after training for OR and 4 h after training for OP to determine a dose of Dkk-1 that did not impair memory consolidation on its own. The 24- and 4-h time points were used because vehicle-treated OVXed females have intact memory for a novel object and moved object, respectively, at these delays (Gresack et al., 2007; Boulware et al., 2013; Fortress et al., 2013; Pereira et al., 2014; Kim et al., 2016; Kim and Frick, 2017). After determining a dose of Dkk-1 that did not impair memory on its own, delays of 48 and 24 h were used subsequently for OR and OP, respectively, because vehicle-infused OVXed females show impaired OR and OP memory, whereas E2-treated OVXed females show intact OR and OP memory, at these delays (Gresack et al., 2007; Boulware et al., 2013; Fortress et al., 2013; Pereira et al., 2014; Kim et al., 2016; Kim and Frick, 2017).
Western blotting

Western blotting was conducted as described previously (Fernandez et al, 2008; Boulware et al, 2013). To determine the effects of Dkk-1 on E2-mediated cell-signaling, mice received infusions as described above and were then cervically dislocated and decapitated 5 or 45 min later. The DH was immediately dissected bilaterally and frozen at -80°C until homogenized. Briefly, tissue samples were resuspended 1:25 w/v in lysis buffer and homogenized using a probe sonicator (Branson Sonifier 250). Homogenates were electrophoresed on TGX (Tris-Glycine eXtended) stain-free precast gels (Bio-Rad) and transferred to polyvinylidene fluoride (PVDF) membranes using the TransBlot Turbo system (Bio-Rad). Membranes were blocked with 5% milk and incubated with primary antibodies (phospho-GSK3β, phospho-ERK, c-myc, phospho-JNK, synaptophysin, phospho-PKC, active β-Catenin 1:1000, Cell Signaling Technology; Wnt7a, 1:1000, abcam) overnight at 4°C. The following day, blots were incubated for 1 h at room temperature with a rabbit HRP-conjugated secondary antibody (1:5000, Cell Signaling Technology), and developed using West Dura chemiluminescent substrate (Pierce). A ChemiDoc MP gel imager (Bio-Rad) was used to detect signal correlating with protein expression, and densitometry was performed using Image Lab software (Bio-Rad). Protein expression was normalized to total membrane-bound protein expression, and data were represented as percent immunoreactivity relative to vehicle controls. Treatment effects were measured within single gels (n=6-8/group).

Data analysis

All statistical analyses were conducted using GraphPad Prism 6 (La Jolla, CA). To determine whether each group demonstrated intact memory consolidation for each task, OR and OP data were first analyzed using one-sample t-tests to determine whether time spent with the
novel or moved object differed significantly from chance (15 s; Boulware et al., 2013; Kim et al., 2016; Tuscher et al., 2016; Kim et al, 2017). This analysis was used because time spent with the objects is not independent; time spent with one object necessarily reduces time spent with the other (Frick and Gresack 2003). To assess between-group treatment effects within each behavioral experiment, one-way ANOVAs with treatment as the independent variable were conducted, followed by Fisher’s LSD post hoc tests. Western blot data were analyzed using one-way ANOVAs followed by Fisher’s LSD post hoc tests and selected t-tests. Statistical significance was determined at $p < 0.05$. Effect sizes were calculated using $\eta^2$ for ANOVAs (sum of squares between/sum of squares total) and Cohen’s d (mean of differences/standard deviation of differences) for pair-wise comparisons.

Results

*Dkk-1 blocks object recognition memory consolidation*

Although we previously established that DH infusion of Dkk-1 prevents memory consolidation in an object recognition task in male mice (Fortress et al, 2013), here we aimed to determine: 1) whether Wnt/β-catenin signaling is necessary for memory consolidation in ovariectomized female mice, and 2) a dose of Dkk-1 that does not impair memory on its own. Identification of a behaviorally subeffective dose of Dkk-1 was necessary to ensure that any impairment seen after Dkk-1+E2 infusion in subsequent experiments resulted from an interaction between Dkk-1 and E2 rather than a general memory-impairing effect of Dkk-1 alone. Young female mice (n = 8-12/group) were OVXed and implanted with cannula aimed at the bilateral DH. Immediately after object recognition training, mice received bilateral DH infusion of vehicle (saline, n=8-10 per group), 1 ng (n=8), 5 ng (n=12), or 10 ng/hemisphere (n=12) of Dkk-1.
Twenty-four hours later, mice receiving vehicle ($t(9) = 2.412, p = 0.0391, d = 0.763; t(8) = 4.704, p = 0.0015, d = 1.569; t(7) = 3.626, p = 0.0084, d = 1.284$), 1 ng ($t(6) = 3.516, p = 0.0126, d = 1.327$), or 5 ng/hemisphere ($t(11) = 2.790, p = 0.0176, d = 0.805$) of Dkk-1 spent significantly more time than chance (15 s) with the novel object, indicating intact OR memory consolidation (Fig 2A). Conversely, mice receiving 10 ng/hemisphere of Dkk-1 did not spend significantly more time than chance with the novel object, indicating impaired OR memory consolidation ($p = 0.1522$). Similar results were observed for OP (Fig 2B). Four hours after training, mice infused with vehicle ($n=9; t(8) = 3.232, p = 0.0120, d = 1.077$) or 5 ng/hemisphere of Dkk-1 ($n=12; t(11) = 2.675, p = 0.0216, d = 0.772$) spent significantly more time than chance with the moved object. These results demonstrate that 5 ng/hemisphere Dkk-1 does not block memory consolidation in either OR or OP, thus, allowing us to use this behaviorally sub effective dose in subsequent

![Fig. 2](image-url) 5ng/hemisphere Dkk-1 does not impair object recognition or object placement memory consolidation. (A) Twenty-four hours after training, mice receiving DH infusion of vehicle, 1ng/hemisphere of Dkk-1, and 5 ng/hemisphere of Dkk-1 spent significantly more time with the novel object than chance (dashed line at 15 s) and than all other groups. (B) Four hours after training, mice receiving DH infusion of vehicle or 5 ng/hemisphere of Dkk-1 spent significantly more time with the novel object than chance (*$p < 0.05$). *$p < 0.05$ relative to chance. Bars represent the mean ± standard error of the mean (SEM).
experiments with E₂.

*Dkk-1 blocks the memory-enhancing effects of E₂ on object recognition and object placement memory consolidation*

To determine whether Wnt/β-catenin signaling is necessary for the memory-enhancing effects of E₂, young female mice were OVXed and implanted with DH and ICV cannulae. Immediately after OR training, mice received bilateral DH infusion of vehicle or 5 ng/hemisphere Dkk-1, followed by ICV infusion of vehicle or E₂ (10 µg). Forty-eight hours later, mice receiving infusions of vehicle + vehicle (n=15), Dkk-1 + vehicle (n=20), or Dkk-1 + E₂ (n=22) spent no more time than chance (15 s) with the novel object, indicating impaired OR memory consolidation. In contrast, mice receiving infusions of vehicle + E₂ (n=20) spent significantly more time with the novel object than chance (dashed line at 15 s) and than all other groups (Veh+Veh, n = 15; Dkk-1+Veh, n = 20, Veh+E₂, n = 20, Dkk-1+E₂, n = 22).

**Fig. 3.** Dkk-1 blocks E₂-mediated memory enhancement. (A) Forty-eight hours after training, mice receiving DH infusion of vehicle and ICV infusion of E₂ spent significantly more time with the novel object than chance (*p < 0.05) and than all other groups (Veh+Veh, n = 18; Dkk-1+Veh, n = 20, Veh+E₂, n = 20, Dkk-1+E₂, n = 22). *p < 0.05 relative to chance; #p < 0.05 relative to the Veh+E₂ group. Bars represent the mean ± standard error of the mean (SEM).
more time than chance with the novel object ($t(19) = 5.358$, $p < 0.0001$, $d = 1.200$; Fig. 3A), demonstrating intact OR memory consolidation. This pattern of significance suggests that Dkk-1 blocked the memory-enhancing effects of E$_2$. This conclusion is supported by the one-way ANOVA, in which the main effect of treatment was significant ($F_{(3,73)} = 3.161$, $p = 0.0296$, $\eta^2 = 0.1149$; Fig 3A) and post hoc tests revealed that mice infused with vehicle+E$_2$ spent significantly more time with the novel object than all other groups. Similar results were observed for OP. Twenty-four hours after training, mice infused with vehicle+vehicle (n=18), Dkk-1+vehicle (n=20), or Dkk-1+E$_2$ (n=22) spent no more time than chance (15 s) with the moved object, whereas mice receiving vehicle+E$_2$ (n=20) spent significantly more time than chance with the moved object ($t(19) = 5.154$, $p < 0.0001$; Fig. 3B). Again, the one-way ANOVA indicated a significant main effect of treatment ($F_{(3,76)} = 3.604$, $p = 0.0171$, $\eta^2 = 0.1246$; Fig 3B) and post hoc tests revealed that mice infused with vehicle+E$_2$ spent significantly more time with the moved object than all other groups. Collectively, these results demonstrate that Dkk-1 prevented E$_2$ from enhancing consolidation in both OR and OP.

**Dkk-1 modulates changes in Wnt/β-catenin signaling proteins**

Our behavioral data indicate a role for Wnt/β-catenin signaling in E$_2$-induced memory consolidation. Therefore, we next examined the effects of E$_2$ and Dkk-1 on Wnt/β-catenin signaling in the DH. Specifically, we hypothesized that E$_2$ would activate Wnt/β-catenin signaling, and Dkk-1 would block this activation. Two weeks after the conclusion of behavioral testing, mice tested above were infused a final time with vehicle+vehicle, Dkk-1+vehicle, vehicle+E$_2$, or Dkk-1+E$_2$ as described above. DH tissue was collected 5 or 45 min later for analysis of total β-catenin, phospho-GSK3β, c-myc, and Wnt7a protein levels. We also assayed
levels of phosho-p42 ERK and phosho-p44 ERK protein as positive and negative controls for E₂-induced activation of hippocampal cell signaling. Our previous work shows that E₂ selectivity increases phosho-p42 ERK levels 5 min after DH or ICV infusion (Fernandez et al., 2008; Boulware et al., 2013; Fortress et al., 2013; Kim et al., 2016). Consistent with these reports, ICV infusion of E₂ increased phosho-p42 ERK levels ($F_{(3,28)} = 4.335, p < 0.05, \eta^2 =$
0.3171; Fig. 4A), but not phospho-p44 ERK levels \( F_{(3,28)} = 0.9495, p = 0.4301; \) data not shown) 5 min post-infusion. This effect was not blocked by DH infusion of Dkk-1, potentially suggesting no E2-induced interaction between the Wnt/β-catenin and ERK signaling pathways. Interestingly, E2 increased phospho-GSK3β levels 45 min post-infusion \( F_{(3,30)} = 3.257, p < .05, \eta^2 = .2457; \) Fig 4C), but this was also not blocked by Dkk-1. Even more peculiarly, E2 had no effect on levels of c-myc 5 min after infusion or on Wnt7a 45 min after infusion. However, the behaviorally sub-effective dose of Dkk-1 used for our behavioral experiments reduced c-myc protein levels sufficiently to produce a main effect of treatment \( F_{(3,25)} = 3.411, p < 0.05, \eta^2 = .2905; \) Fig 4B), in which levels were significantly reduced relative to vehicle+vehicle. A similar pattern was evident for Wnt7a protein 45 min post infusion but the main effect of treatment was not significant \( F_{(3,20)} = 1.483, p = .2494; \) Fig 4D). Importantly, levels of β-catenin protein remained unchanged in any treatment group at either time point (data not shown). Together, these data suggest only a modest activation of Wnt/β-catenin signaling by E2, but curiously, indicate that Dkk-1 may not block E2-induced memory enhancement in a β-catenin-dependent manner.

**Dkk-1 modulates changes in non-canonical Wnt signaling proteins**

Given the above findings, we expanded our search of biochemical changes underlying the above-described behavioral effects to include targets of β-catenin-independent pathways, Wnt/JNK, and Wnt/Calcium signaling. We examined levels of phosphorylated p46 JNK protein and a downstream target of Wnt/JNK activity, synaptophysin. In addition, we assessed levels of phosphorylated PKC protein, a marker of Wnt/Calcium signaling. Our rationale for examining these pathways comes from evidence suggesting that Dkk-1 may activate Wnt/JNK signaling (Kilick et al, 2014; Lee et al, 2004), as well as data indicating a potential role for noncanonical
Wnt signaling in antagonizing Wnt/β-catenin signaling (Topol et al, 2003). The main effect of treatment was significant for phospho-p46 JNK ($F_{(3,32)} = 5.069$, $p < 0.01$, $\eta^2 = .3222$; Fig 5A), such that levels 5 min post-infusion were significantly increased relative to vehicle+vehicle in both groups receiving Dkk-1. Synaptophysin levels were significantly affected by treatment ($F_{(3,28)} = 5.250$, $p < 0.01$, $\eta^2 = .3600$; Fig 5B), such that infusion of Dkk-1+vehicle significantly

**Fig. 5.** Expression of Wnt/JNK signaling proteins is influenced 5 min after Dkk-1 and E₂ infusion. (A) Dorsal hippocampal infusion of Dkk-1 increased levels of phospho-JNK 5 min after infusion, regardless of whether vehicle or E₂ was infused ICV. (B) Synaptophysin levels were decreased 5 min after DH infusion of Dkk-1. (C) Levels of phospho-PKC were increased 5 min after ICV infusion of E₂, regardless of whether vehicle or Dkk-1 was infused into the DH. Each bar represents the mean ± SEM percent change from vehicle (*$p < 0.05$).
decreased synaptophysin levels relative to vehicle 45 min post-infusion ($p < 0.05$). E$_2$ appeared to mitigate this decrease, as levels in the Dkk-1+E$_2$ group did not differ from vehicle. Phosphorylated PKC protein expression was significantly increased 5 min following E$_2$ infusion ($F(3,18) = 3.381, p < 0.05; \eta^2 = .3604$, Fig 5C), an effect which was not blocked by Dkk-1. These data indicate that Dkk-1 may interface with Wnt/JNK signaling in the hippocampus, but not with Wnt/Calcium signaling.

**Discussion**

Previous work from our lab and others has demonstrated that Wnt/β-catenin signaling plays a critical role in hippocampal memory processes (Fortress et al, 2013; Maguschak and Ressler, 2011). Moreover, a role for E$_2$ in rapidly activating cell signaling within the DH to mediate memory consolidation has been repeatedly reported (Fernandez et al, 2008; Fan et al, 2010). However, the present study is the first to demonstrate that E$_2$-mediated memory enhancement involves Wnt signaling. First, we demonstrated that DH infusion of the endogenous Wnt signaling antagonist Dkk-1 prevents E$_2$-mediated memory enhancement. Second, we showed that this behavioral effect is accompanied by suppression of specific elements of Wnt/β-catenin signaling, and activation of Wnt/JNK signaling. Collectively, our results provide novel insight into the pathways through which E$_2$ works to mediate memory processes within the female mouse DH.

Our data demonstrating that DH infusion of Dkk-1 blocks the memory-enhancing effects of E$_2$ in OVXed mice is consistent with and extends our previous report that Dkk-1 prevents object recognition memory consolidation in male mice (Fortress et al, 2013). Our previous work involved DH infusion of a 10-fold higher dose (50 ng/hemisphere) of Dkk-1 than the current
study. Here, we aimed to determine whether inhibition of Wnt/β-catenin signaling could block the memory enhancing effects of E2. In our first experiment, we found that even a 10ng/hemisphere dose of Dkk-1 could impair object recognition memory consolidation. Therefore, we used a lower dose of Dkk-1 no effects on memory (5 ng/hemisphere) to determine whether Dkk-1 interferes with the memory-enhancing effects of E2. As predicted, Dkk-1 did prevent E2 from enhancing both object recognition and object placement memory consolidation. This result is consistent with data demonstrating that ICV injection of Dkk-1 reverses the neuroprotective effects of E2 in the female hippocampus (Scott and Brann, 2013), and extend these findings to show a novel role for Dkk-1 in preventing E2-induced memory enhancement among normal females.

Because Dkk-1 is a known antagonist of Wnt/β-catenin signaling, we hypothesized that it blocked the memory-enhancing effects of E2 by turning “off” Wnt/β-catenin signaling, and thus would prevent E2 from increasing hippocampal levels of phospho-GSK3β, β-catenin, c-myc, and Wnt7a protein. However, we were surprised to find only modest suppression of Wnt/β-catenin signaling proteins. Although our data indicating that DH infusion of Dkk-1 suppresses levels of c-myc and Wnt7a proteins are consistent with our hypothesis, there was no E2-mediated increase in these proteins, suggesting that Dkk-1 may actually block memory consolidation through alternative, non-canonical pathways, and that E2 may not robustly activate Wnt/β-catenin signaling as expected. Indeed, the lack of E2-mediated increases in Wnt/β-catenin signaling proteins, with the exception of phosphorylated GSK3β, suggests that E2 may not enhance memory through direct activation of Wnt/β-catenin signaling. Our data describing an E2-mediated increase in phosphorylated GSK3β protein are consistent with literature demonstrating a role for E2 in increasing activation of GSK3β (Cardona-Gomez, 2004; Varea et al, 2009).
However, GSK3β may interface with additional cell signaling pathways that are involved in E2-mediated memory enhancement, such as the PI3K pathway. The idea that GSK3β plays multifaceted roles in cell-signaling activity, which is well supported by evidence suggesting that GSK3β is a ubiquitous kinase that interfaces with PI3K, Akt, and mTOR (for review, see Hermida et al., 2017), merits further research. Apart from its described role in Wnt/β-catenin signaling, GSK3β is reported to have over 100 identified substrates (Sutherland, 2011), and is predicted to interact with many more. Consistent with this idea, researchers have posited that there are multiple available pools of GSK3β that are both physically and functionally distinct, such that activation of GSK3β does not necessarily result in activation of Wnt/β-catenin signaling (Beurel et al., 2015; Wu and Pan, 2010). Thus, E2-mediated increases in phosphorylated GSK3β may occur independently of Wnt/β-catenin signaling activity.

The observed effects of Dkk-1 on levels of Wnt/β-catenin signaling proteins, especially compared to the robust effects of Dkk-1 on these proteins we previously observed (Fortress et al., 2013), may be due to our use of a lower, behaviorally subeffective dose of Dkk-1, or examination of time points at which Dkk-1 does not most strongly exert its effects. We chose to examine changes in protein expression 5 min after infusion based on our previous work showing that E2 activates numerous other kinases within 5 min (Fernandez et al., 2008; Fortress et al., 2013), but we were surprised to find limited effects on Wnt/β-catenin signaling. Inclusion of a 45 min time point was guided by data from our lab and others showing the effects of E2 on GSK3β and β-catenin activity occurring largely within a 1 h window (Cardona-Gomez et al., 2004; Fortress et al., 2013). Examining more extended time points in future work may aid in characterizing the interactions among E2, Dkk-1, and Wnt/β-catenin signaling. Despite our surprisingly modest biochemical effects on Wnt/β-catenin proteins, our behavioral data
concretely demonstrate that this dose of Dkk-1 can block the memory-enhancing effects of E₂. Importantly, we found no effects of Dkk-1 infusion or E₂ infusion on levels of β-catenin (data not shown), which is a primary downstream effector of Wnt/β-catenin signaling. Thus, we further hypothesized that Dkk-1 may act through alternative non-canonical Wnt pathways to block the effects of E₂ on memory consolidation.

Despite the general consensus that Dkk-1 antagonizes Wnt/β-catenin signaling, multiple recent studies have suggested more diverse roles for Dkk-1. Thus, we chose to examine Wnt/JNK signaling, a pathway originally identified for its role in regulating epithelial cell polarity in *Drosophila* and gastrulation in vertebrates (Boutros et al, 1998, Sokol 2000). Although to the best of our knowledge, no published studies have demonstrated a role for Wnt/JNK signaling in memory processes, Dkk-1 appears to promote synapse disassembly by concurrent blockade of Wnt/β-catenin signaling and activation of RhoA-Rock signaling, both elements of the Wnt/JNK cascade (Marzo et al., 2016). We chose to examine levels of phosphorylated JNK protein and the synaptic vesicle membrane protein synaptophysin because both JNK and synaptophysin may play a role in synaptic plasticity, and in turn, affect memory processes (Mao and Wang, 2016; Tarsa and Goda, 2002).

We found that DH infusion of Dkk-1 increases phosphorylated JNK protein in the DH, whereas ICV infusion of E₂ has no effect on levels of this protein. Furthermore, DH infusion of Dkk-1 decreased synaptophysin protein levels; this decrease was mitigated by ICV infusion of E₂. Combined, these data suggest that Dkk-1 may exert non-canonical effects in the DH through Wnt/JNK signaling. Our data are consistent with other research demonstrating a β-catenin-independent, and JNK-dependent, role for Dkk-1. For example, in human mesothelioma cell lines deficient in β-catenin, Dkk-1 still acts as a tumor suppressor, indicating that Dkk-1 can act
in a β-catenin independent manner (Lee et al, 2004). Evidence from studies using *Xenopus* and zebrafish also suggests that Dkk-1 can operate independently of β-catenin to regulate gastrulation through Wnt/JNK signaling, as evidenced by a robust increase in JNK phosphorylation (Caneparo et al, 2007). Additionally, in an Alzheimer’s disease mouse model, transcriptional upregulation of genes that mediate the development of Aβ pathology appears to be primarily driven by Dkk-1-mediated activation of Wnt/JNK signaling (Killick et al, 2014). Our data showing a decrease in levels of synaptophysin protein and increase in levels of phosphorylated JNK mediated by Dkk-1 are supported by the finding that Dkk-1 promotes synapse disassembly through a JNK-dependent mechanism (Marzo et al, 2016), and the finding that synaptophysin plays a functional role in regulating activity-dependent synapse formation (Tarsa and Goda, 2002). Moreover, in the present study, we demonstrated modest effects of Dkk-1 on suppressing Wnt/β-catenin signaling in combination with our finding of Dkk-1 mediated JNK activation, supporting the hypothesis that Dkk-1 may act bidirectionally to suppress canonical Wnt activity while enhancing Wnt/JNK activity. These data, in combination with those from the current study, suggest a novel role for Dkk-1 in altering Wnt/JNK signaling.

We also examined Wnt/Calcium signaling and its downstream effector PKC, given that in addition to its role in regulating cell adhesion and motility, Wnt/Calcium signaling also appears to be critical for dendritic arborization and synaptic plasticity (Cerpa et al, 2011; Chen et al, 2017; Kuhl et al. 2000). We demonstrated in the present study that E2 increases phosphorylated PKC protein, but that Dkk-1 does not mitigate this increase. E2 may increase levels of phosphorylated PKC independent of Wnt/Calcium signaling activity; indeed, E2-induced increases in phosphorylated PKC are well documented. Given that infusion of Dkk-1 into the DH does not independently alter levels of phosphorylated PKC, the current study
suggests that Dkk-1 may signal through JNK, but not through the Wnt/Calcium signaling pathway, to impair memory consolidation. These data lend credence to the idea that Dkk-1 may act in a bidirectional fashion to block Wnt/β-catenin signaling while also simultaneously activating Wnt/JNK signaling in the DH, and suggest that Dkk-1 operates independently of the Wnt/Calcium signaling pathway. Future studies must take into account that Dkk-1 may not specifically interact with Wnt/β-catenin signaling as previously thought, and more work remains to pinpoint the precise roles of Dkk-1 in mediating Wnt/JNK signaling.

The current study suggests a novel role for Dkk-1 in preventing the E₂-induced enhancement of memory consolidation via activation of JNK signaling. Recent data from our lab posit that a specific estrogen receptor, GPER, enhances memory through activation of JNK signaling, rather than ERK signaling (Kim et al, 2016). Although the current data contradict the idea that JNK activation enhances memory consolidation, the interactions between Dkk-1 and E₂ may depend on its interaction with other estrogen receptors in the hippocampus, such as ERα or ERβ, rather than GPER. Indeed, the memory-enhancing effects of E₂ appear to activate cell-signaling cascades independent of GPER, given that GPER activation within the DH is not necessary for E₂ to enhance memory (Kim et al, 2016). Future work should investigate the potential estrogen receptor mechanisms mediating the interaction between E₂ and Dkk-1.

In conclusion, the present study demonstrates that Dkk-1 infusion into the DH block the effects of E₂ on object recognition and spatial memory consolidation in OVXed mice. Although much more work remains to identify the precise mechanism through which this interaction occurs, the present study suggests that Dkk-1 might operate bi-directionally to inhibit Wnt/β-catenin signaling while also promoting Wnt/JNK signaling in the DH. These findings provide novel insight into the ways in which Dkk-1 acts within the DH, and contribute to a growing body
of literature indicating that Dkk-1 may function in additional capacities that were not previously appreciated. Our findings implicate novel pathways involved in the memory-enhancing effects of E2.
References


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