Hormones and Behavior 114 (2019) 104545

Contents lists available at ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh

Dickkopf-1 blocks 17β-estradiol-enhanced object memory consolidation in ovariectomized female mice

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ARTICLE INFO

Keywords:
17β-estradiol
Object recognition
Object placement
Wnt signaling
Mouse
Dkk-1
JNK

ABSTRACT

The memory-enhancing effects of 17β-estradiol (E_2) depend upon rapid activation of several cell-signaling cascades within the dorsal hippocampus (DH). Among the many cell-signaling pathways that mediate memory processes, Wnt/β-catenin signaling has emerged as a potential key player because of its importance to hippocampal development and synaptic plasticity. However, whether E_2 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 E_2-induced memory consolidation in ovariectomized mice tested in the object recognition and object placement tasks. Ovariectomized C57BL/6 mice received immediate post-training infusions of E_2 or vehicle into the dorsal third ventricle plus the endogenous Wnt/β-catenin antagonist Dickkopf-1 (Dkk-1) or vehicle into the DH to assess whether the memory-enhancing effects of E_2 depend on activation of Wnt/β-catenin signaling. Our results suggest that Dkk-1 blocks E_2-induced memory enhancement as hypothesized, but may do so by only moderately blunting Wnt/β-catenin signaling while concurrently activating Wnt/JNK signaling. The current study provides novel insights into the mechanisms through which E_2 enhances memory consolidation in the DH, as well as critical information about the mechanistic actions of Dkk-1.

1. Introduction

The sex steroid hormone 17β-estradiol (E_2) 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 molecular mechanisms underlying E_2-enhanced memory formation remain to be fully characterized. Infusion of E_2 into the dorsal hippocampus (DH) of ovariectomized (OVXed) mice rapidly triggers several cell-signaling cascades, including the extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K), and mammalian target of rapamycin (mTOR)-mediated pathways (Fernandez et al., 2008; Fan et al., 2010; Fortress et al., 2013a). Moreover, this activation is necessary for E_2 to facilitate object recognition and spatial memory consolidation in OVXed mice (Boulware et al., 2013; Fernandez et al., 2008; Fan et al., 2010; Kim et al., 2016), illustrating that rapid initiation of DH cell signaling plays an essential role in the memory-enhancing effects of E_2.

Among other key signaling pathways that may also regulate the effects of E_2 on memory is Wnt/β-Catenin (aka, canonical Wnt) signaling. Wnt/β-Catenin signaling is one of three primary Wnt signaling pathways, and is implicated in hippocampal development and plasticity (for review, see Budnik and Salinas, 2011; Fortress and Frick, 2016; Inestrosa and Arenas, 2010; Komiya and Habas, 2008; Scott and Brann, 2013). 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 transcription factors such as TCF or LEF, ultimately increasing expression of target genes including *Cyclin D1* and *c-myc* (Logan and Nusse, 2004). In the absence of Wnt ligand binding, GSK3β phosphorylates β-catenin, allowing for ubiquitination and targeting of β-catenin for proteosomal degradation (Logan and Nusse, 2004). Wnt/β-catenin signaling can be blocked by Dickkopf-1 (Dkk-1), an endogenous protein that prevents Wnt ligands from complexing with Fz and LRP5/6 co-receptors and ultimately prevents β-catenin-induced gene transcription (Bafico et al., 2001; Inestrosa and Arenas, 2010).

Increasing evidence suggests that Wnt/β-catenin signaling plays an important role in learning and memory processes, especially within the hippocampus. In male rodents, canonical Wnt ligands like Wnt7a/b are trafficked to hippocampal dendrites after glutamate activation

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https://doi.org/10.1016/j.yhbeh.2019.06.009
Received 25 March 2019; Received in revised form 15 June 2019; Accepted 16 June 2019
Available online 12 August 2019

(Tabatadze et al., 2014) and are increased in the hippocampus after spatial or object learning (Tabatadze et al., 2012; Fortress et al., 2013b). Similarly, contextual fear conditioning increases hippocampal expression of canonical Wnt3a in male mice (Xu et al., 2015). These increases have functional consequences, as inhibition of Wnt signaling via intrahippocampal infusion of a Wnt3a antibody or of Dkk-1 impairs contextual fear conditioning and object recognition memory consolidation, respectively, in male mice (Xu et al., 2015; Fortress et al., 2013b). In contrast, activating Wnt signaling in adult mice with Wnt-activating small molecule (WASP-1) enhances episodic memory, and concurrently increases basal excitatory synaptic transmission and facilitates LTP (Vargas et al., 2014). Combined, these data support the critical involvement of Wnt/β-catenin signaling in hippocampal memory formation among male rodents.

However, the putative role of Wnt/β-Catenin signaling in mediating memory in female rodents is unknown. Evidence from multiple studies suggests that E_2 activates Wnt/β-catenin signaling in females, particularly in models of ischemic brain injury (Zhang et al., 2008; Scott and Brann, 2013). In primary hippocampal and cortical cultures, E_2 reduces GSK3β activity, increases the amount and stability of β-catenin, and increases β-catenin-mediated gene transcription (Cardona-Gomez et al., 2004; Varea et al., 2009). Similar increases in GSK3β phosphorylation, indicative of reduced GSK3β activity, have been reported in OVXed rats treated with E_2 (Cardona-Gomez et al., 2004). Although these data suggest a role for E_2 in mediating Wnt/β-catenin signaling, evidence describing the extent to which activation of this pathway is necessary for E_2 to promote memory consolidation is lacking. Therefore, the present study investigated whether Wnt/β-catenin signaling is necessary for the memory-enhancing effects of E_2 previously observed in OVXed mice (Fernandez et al., 2008; Fortress et al., 2013a; 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 E_2. Our findings suggest that Dkk-1 prevents E_2 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.

2. Materials and methods

2.1. 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*.

2.2. 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.

2.3. Surgery

All surgeries were conducted starting three days after arrival in the laboratory as described previously (Boulware et al., 2013; Fortress et al., 2013a, 2013b). 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., 2013a). 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 E_2 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 also 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 to recover prior to the start of behavioral testing.

2.4. Drugs and infusions

The Wnt/β-catenin inhibitor Dickkopf-1 (Dkk-1) was dissolved in sterile 0.9% saline to concentrations of 2, 10, or 20 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, resulting in doses of 1, 5, or 10 ng/μl. Cyclodextrin-encapsulated E_2 (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile 0.9% saline to a concentration of 10 μg/μl. The vehicle consisted of 2-hydoxypropyl-β-cyclodextrin (HBC; Sigma-Aldrich, St. Louis, MO) dissolved in saline to the same concentration of cyclodextrin present in the cyclodextrin-encapsulated E_2 solution. A total volume of 1 μl E_2 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 laboratory to prevent potential damage to the DH from two infusions into the DH in rapid succession, 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 (Fernandez et al., 2008; Fan et al., 2010; Zhao et al., 2010, 2012; Boulware et al., 2013; Fortress et al., 2013a).

2.5. Behavioral testing

Object recognition (OR) and object placement (OP) were used to measure object recognition and spatial memory as we have described previously (Fernandez et al., 2008; Fortress et al., 2013a, 2013b; Boulware et al., 2013; Kim et al., 2016; Tuscher et al., 2016). Mice were handled for 30 s/day for three days prior to habituation. On the second day of handing, a Lego Duplo block was placed in each home cage to habituate mice to objects during the remaining days of handling and habituation. After three days of handling, mice were habituated to the empty testing apparatus (60 cm × 60 cm × 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 s 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 (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). Object exploration was scored when the mouse was immediately adjacent to an object and its nose and/or front paws were directed at or touched the object. Exploration was experimenter-defined in real time by experienced raters who entered this information into ANYmaze for quantification.

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., 2013a, 2013b; 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, longer delays of 48 and 24 h were used subsequently for OR and OP, respectively, to test whether Dkk-1 could block E_2-induced enhancements in each task. At these delays, vehicle-infused OVXed females fail to show intact OR and OP memory, whereas E_2-treated OVXed females exhibit enhanced OR and OP memory (Gresack et al., 2007; Boulware et al., 2013; Fortress et al., 2013a, 2013b; Pereira et al., 2014; Kim et al., 2016; Kim and Frick, 2017). Thus, these delays are appropriate to test whether antagonists such as Dkk-1 can prevent E_2 from enhancing memory consolidation.

2.6. Western blotting

Western blotting was conducted as described previously (Fernandez et al., 2008; Boulware et al., 2013; Fortress et al., 2013a, 2013b). To determine the effects of Dkk-1 on E_2-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). Total protein was then imaged on the membranes using a ChemiDoc MP gel imager (Bio-Rad), and a lane profile containing total protein density measurements was created in the Image Lab software (Bio-Rad) for each homogenate and saved for later analysis and normalization. Membranes were then blocked with 5% milk and incubated with primary antibodies (phospho-GSK3β, #9336, phospho-ERK, #9101, c-myc, #5605, phospho-JNK, #9251, synaptophysin, #5461, phospho-PKC, #9375, active β-Catenin, #19807, Cell Signaling Technology; Wnt7a, ab100792, abcam) overnight at 4 °C. All primary antibodies were used at a concentration of 1:1000, with the exception of phospho-ERK, which was used at a concentration of 1:3000. 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). The ChemiDoc MP gel imager was used to detect signal correlated with protein expression, and densitometry was performed using Image Lab software. Protein expression was then normalized to the total protein expression measured prior to antibody incubation. Data were represented as percent immunoreactivity relative to vehicle controls. Treatment effects were measured within single gels (n = 6–8/group).

2.7. 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 and Frick, 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. To assess whether post-training drug treatments influenced activity during testing, the time for each mouse to accumulate 30 s of exploration was collected and analyzed with one-way ANOVAs and Student's t-tests where appropriate. Western blot data were analyzed using one-way ANOVAs followed by Fisher's LSD post hoc tests. Statistical significance was determined at $p < 0.05$. Effect sizes were calculated using η^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.

3. Results

3.1. Dkk-1 blocks object recognition and object placement 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., 2013b), 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 sub-effective dose of Dkk-1 was necessary to ensure that any impairment seen after Dkk-1 + E_2 infusion in subsequent experiments resulted from an interaction between Dkk-1 and E_2 rather than a general memory-impairing effect of Dkk-1 alone. Young female mice (n = 8–12/group) were OVXed and implanted bilaterally with cannulae aimed at the DH. Immediately after object training, mice received bilateral DH infusion of vehicle (saline, n = 8–10 per group), 1 ng/hemisphere (n = 8), 5 ng/hemisphere (n = 12), or 10 ng/hemisphere (n = 12) of Dkk-1. Twenty-four hours later, mice receiving vehicle ($t_{(9)} = 2.41$, $p = 0.0391$, d = 0.76; $t_{(8)} = 4.70$, $p = 0.0015$, d = 1.57; $t_{(7)} = 3.63$, $p = 0.0084$, d = 1.28), 1 ng ($t_{(6)} = 3.52$, $p = 0.0126$, d = 1.33), or 5 ng/hemisphere ($t_{(11)} = 2.79$, $p = 0.0176$, d = 0.81) of Dkk-1 spent significantly more time than chance (15 s) with the novel object, indicating intact OR memory consolidation (Fig. 1A). Conversely, mice receiving 10 ng/hemisphere of Dkk-1 did not spend significantly more time than chance with the novel object ($p = 0.1522$), indicating impaired OR memory consolidation. Importantly, time (in seconds) to accumulate 30 s of object exploration did not differ 24 h post-infusion among mice treated with vehicle (M = 599.7, SEM = 51.81), 1 ng (M = 560.5, SEM = 59.64), 5 ng (M = 533.2, SEM = 86.22), or 10 ng/hemisphere Dkk-1 (M = 536.5, SEM = 70.64; $F_{(3,56)} = 0.26$, $p = 0.8518$), suggesting that these doses of Dkk-1 do not interfere with total activity during testing. Because 5 ng/hemisphere was the highest dose that did not impair OR memory consolidation, we then tested its effects in OP. Similar results were observed for OP (Fig. 1B). Four hours after training, mice infused with vehicle (n = 9; $t_{(8)} = 3.23$, $p = 0.012$,

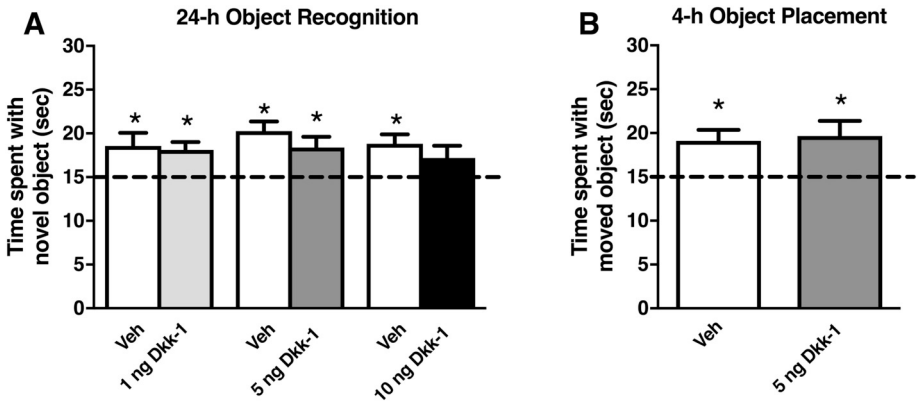

Fig. 1. Object recognition memory was impaired in OVXed females by 10 ng/hemisphere Dkk-1, but neither object recognition nor object placement was impaired by 5 ng/hemisphere Dkk-1. (A) Twenty-four hours after object recognition training, mice receiving DH infusion of vehicle, 1 ng/hemisphere Dkk-1, or 5 ng/hemisphere Dkk-1 spent significantly more time with the novel object than chance (dashed line at 15 s), but mice receiving 10 ng/hemisphere did not. These data suggest that 10 ng/hemisphere Dkk-1 impairs object recognition memory consolidation, but 1 and 5 ng/hemisphere do not. (B) Four hours after object placement training, mice receiving DH infusion of vehicle or 5 ng/hemisphere Dkk-1 spent significantly more time with the moved object than chance, suggesting that this dose also does not impair spatial memory consolidation. *$p < 0.05$ relative to chance. Bars represent the mean ± standard error of the mean (SEM).

d = 1.08) or 5 ng/hemisphere of Dkk-1 (n = 12; $t_{(11)} = 2.68$, $p = 0.0216$, d = 0.78) spent significantly more time than chance with the moved object. Time to accumulate 30 s of object exploration did not differ 4 h ($t_{(8)} = 2.08$, $p = 0.9394$) post-infusion between mice treated with vehicle (M = 290.5, SEM = 31.75) or 5 ng/hemisphere Dkk-1 (M = 286.4, SEM = 39.66), demonstrating that this dose administered post-training did not interfere with non-mnemonic aspects of performance during OP testing. Together, these results demonstrate that 5 ng/hemisphere Dkk-1 does not block memory consolidation in either OR or OP, thus, allowing this behaviorally sub-effective dose to be used in subsequent experiments with E_2.

3.2. Dkk-1 blocks the memory-enhancing effects of E_2 on object recognition and object placement memory consolidation

To determine whether Wnt/β-catenin signaling is necessary for the memory-enhancing effects of E_2, 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 E_2 (10 μg). Forty-eight hours later, mice receiving infusions of vehicle + vehicle ($n = 15$; $t_{(14)} = 0.1$, $p = 0.9187$), Dkk-1 + vehicle ($n = 20$; $t_{(19)} = 0.06$, $p = 0.954$), or Dkk-1 + E_2 ($n = 22$; $t_{(21)} = 0.18$, $p = 0.8595$) spent no more time than chance (15 s) with the novel object, indicating impaired OR memory consolidation. In contrast, mice receiving infusions of vehicle + E_2 (n = 20) spent significantly more time than chance with the novel object ($t_{(19)} = 5.36$, $p < 0.0001$, d = 1.20; Fig. 2A), 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 a one-way ANOVA, in which the main effect of treatment was significant ($F_{(3,73)} = 3.16$, $p = 0.0296$, $\eta^2 = 0.11$; Fig. 2A), and post hoc tests revealing that mice infused with vehicle + E_2 spent significantly more time with the novel object than all other groups ($p = 0.0174$ vs vehicle + vehicle; $p = 0.0119$ vs Dkk-1 + vehicle; and $p = 0.0163$ vs Dkk-1 + E_2). Time to accumulate 30 s of investigatory behavior with objects did not differ among vehicle + vehicle (M = 575.5, SEM = 68.56), Dkk-1 + vehicle (M = 536, SEM = 44.36), vehicle + E_2, (M = 602.9, SEM = 58.31), and Dkk1 + E_2 (M = 587.4, SEM = 48.31) treatment groups 48 h post-infusion ($F_{(3,\ 73)} = 0.21$, $p = 0.8859$), suggesting that drug infusions did not alter total activity 48 h after they were given. Similar results were observed for OP. Twenty-four hours after training, mice infused with vehicle + vehicle ($n = 18$; $t_{(17)} = 0.56$, $p = 0.5845$), Dkk-1 + vehicle (n = 20; $t_{(19)} = 0.79$, $p = 0.4401$), or Dkk-1 + E_2 (n = 22; $t_{(21)} = 0.45$, $p = 0.6551$) 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 ($t_{(19)} = 5.15$, $p < 0.0001$; Fig. 2B). Again, a one-way ANOVA indicated a significant main effect of treatment ($F_{(3,76)} = 3.60$, $p = 0.0171$, $\eta^2 = 0.12$; Fig. 2B), with post hoc tests revealing that mice infused with vehicle + E_2 spent significantly more time with the moved object than all other groups ($p = 0.0275$ vs vehicle + vehicle; $p = 0.0320$ vs Dkk-1 + vehicle; and $p = 0.0022$ vs Dkk-1 + E_2). Again, time to accumulate 30 s of object exploration did not differ among vehicle + vehicle (M = 536.3, SEM = 35.41), Dkk-1 + vehicle (M = 503.2, SEM = 58.38), vehicle + E_2 (M = 613.1, SEM = 51.79), and Dkk-1 + E_2 (M = 552.4, SEM = 45.3) treatment groups 24 h post-infusion ($F_{(3,76)} = 0.88$, $p = 0.4553$). Collectively, these results demonstrate that Dkk-1 prevented E_2 from enhancing consolidation in both OR and OP.

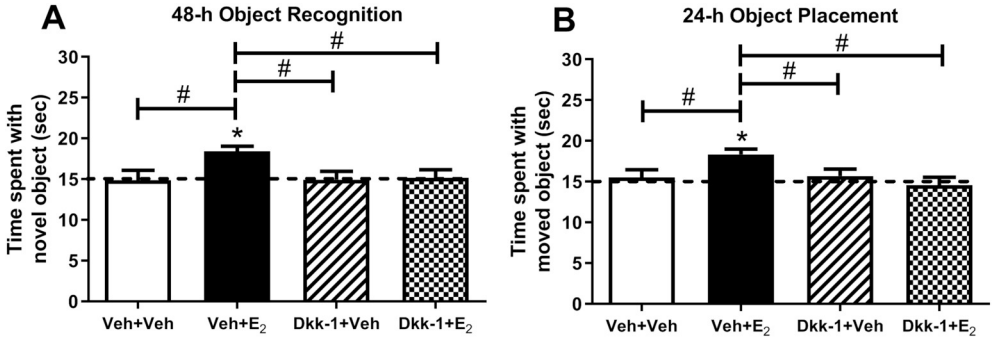

Fig. 2. Dkk-1 blocked E_2-mediated memory enhancement. (A) Forty-eight hours after training, mice receiving DH infusion of vehicle and ICV infusion of E_2 spent significantly more time with the novel object than chance (dashed line at 15 s) and than all other groups (Veh + Veh, $n = 15$; Dkk-1 + Veh, $n = 20$, Veh + E_2, n = 20, Dkk-1 + E_2, $n = 22$). (B) Twenty-four hours after training, mice receiving Veh + E_2 spent significantly more time with the novel object than chance and than all other groups (Veh + Veh, $n = 18$; Dkk-1 + Veh, n = 20, Veh + E_2, n = 20, Dkk-1 + E_2, n = 22). *$p < 0.05$ relative to chance; #$p < 0.05$ relative to the Veh + E_2 group. Bars represent the mean ± standard error of the mean (SEM).

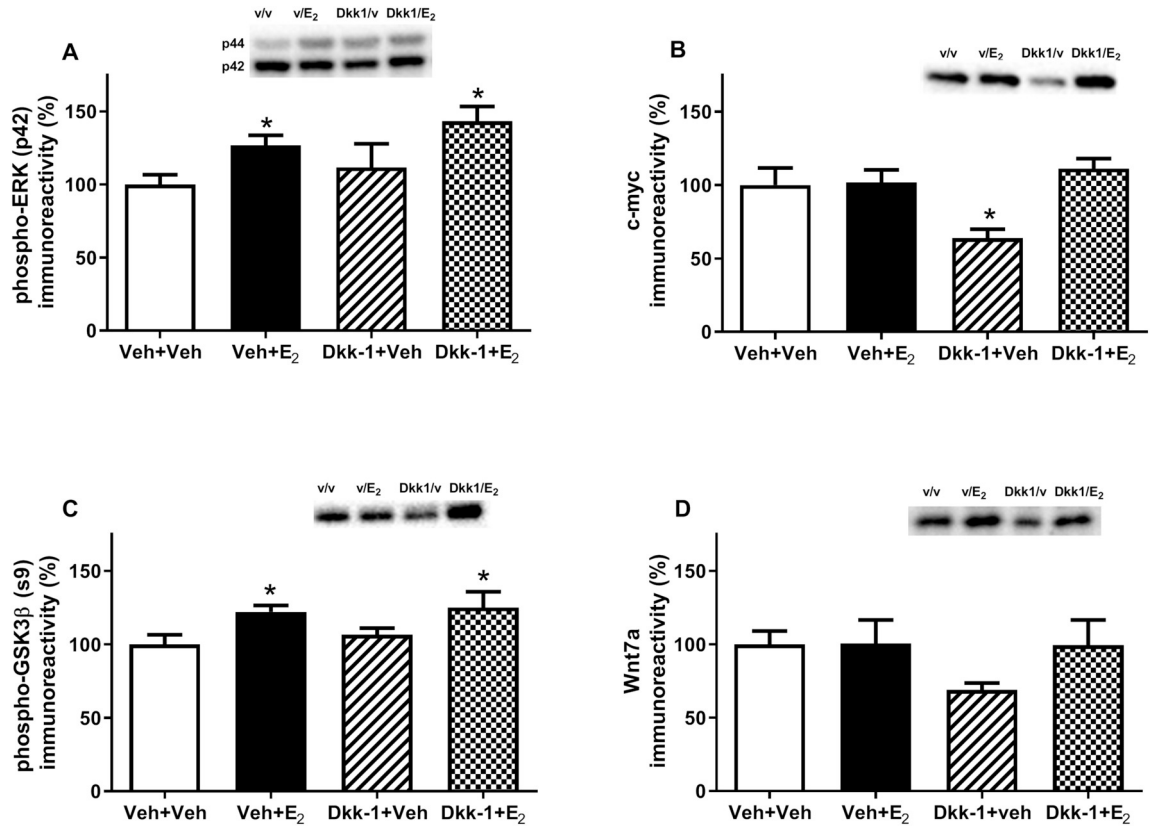

Fig. 3. Expression of Wnt/β-catenin signaling proteins was influenced 5 min (A, B) and 45 min (C, D) following Dkk-1 and E_2 infusion. (A) Intracerebroventricular (ICV) infusion of E_2 (5 μg/hemisphere) increased levels of phosphorylated p42 ERK 5 min post-infusion, an effect that was not blocked by DH infusion of Dkk-1. (B) c-myc levels were reduced 5 min following DH infusion of Dkk-1. (C) Levels of phosphorylated GSK3β were increased 45 min after ICV infusion of E_2, an effect not blocked by DH infusion of Dkk-1. (D) Levels of Wnt7a protein were decreased 45 min after DH infusion of Dkk-1. Each bar represents the mean ± SEM percent change from vehicle (*$p < 0.05$).

3.3. 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, the mice tested in the previous experiment 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. Levels of phospho-p42 ERK and phospho-p44 ERK protein were also assayed as positive and negative controls, respectively, for E_2-induced activation of hippocampal cell signaling. In our previous work, E_2 selectively increased phospho-p42 ERK levels 5 min after DH or ICV infusion (Fernandez et al., 2008; Boulware et al., 2013; Fortress et al., 2013a; Kim et al., 2016). Consistent with these reports, ICV infusion of E_2 increased phospho-p42 ERK levels ($F_{(3,28)} = 4.34$, $p < 0.05$, $\eta^2 = 0.32$; Fig. 3A), but not phospho-p44 ERK levels ($F_{(3,28)} = 0.95$, $p = 0.43$) 5 min post-infusion. This effect was not blocked by DH infusion of Dkk-1, potentially suggesting no E_2-induced interaction between the Wnt/β-catenin and ERK signaling pathways. Interestingly, E_2 increased phospho-GSK3β levels 45 min post-infusion ($F_{(3,30)} = 3.26$, $p < 0.05$, $\eta^2 = 0.25$; Fig. 3C), but this was also not blocked by Dkk-1, such that the vehicle + E_2 group was significantly different from vehicle + vehicle ($p = 0.0231$), as was the Dkk-1 + E_2 group ($p = 0.0143$). Even more peculiarly, E_2 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 ($F_{(3,25)} = 3.41$, $p < 0.05$, $\eta^2 = 0.29$; Fig. 3B), in which levels were significantly reduced relative to vehicle + vehicle ($p = 0.0208$). A similar pattern was evident for Wnt7a protein 45 min post infusion, but the main effect of treatment was not significant ($F_{(3,20)} = 1.48$, $p = 0.2494$; Fig. 3D). Importantly, levels of β-catenin protein remained unchanged in any treatment group at either time point (for 5 min: $F_{(3,20)} = 0.1893$, $p = 0.9025$; for 45 min: $F_{(3,20)} = 1.05$, $p = 0.39$). Together, these data suggest only a modest activation of Wnt/β-catenin signaling by E_2, but curiously, indicate that Dkk-1 may not block E_2-induced memory enhancement in a β-catenin-dependent manner.

3.4. 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 Wnt 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 (Killick et al., 2014; Lee et al., 2004), as well as data indicating a potential role for non-canonical Wnt signaling in antagonizing Wnt/β-catenin signaling (Topol et al., 2003). The main effect of treatment was significant for phospho-p46 JNK ($F_{(3,32)} = 5.07$, $p < 0.01$, $\eta^2 = 0.32$; Fig. 4A), such that levels 5 min post-infusion were significantly increased relative to vehicle + vehicle in both the Dkk-1 + vehicle group ($p = 0.0227$) and the Dkk-1 + E_2 group ($p = 0.0009$). Synaptophysin levels were significantly affected by

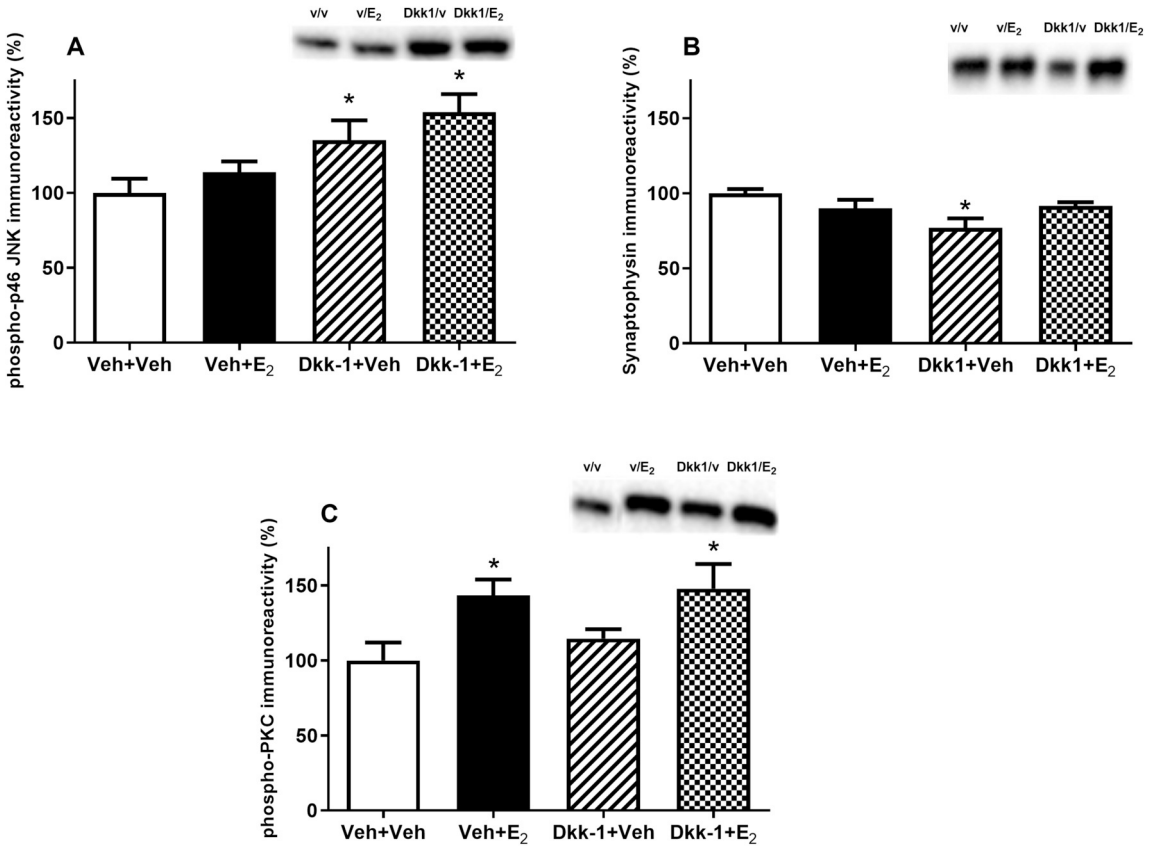

Fig. 4. Expression of Wnt/JNK signaling proteins was influenced 5 min after Dkk-1 and E_2 infusion. (A) Dorsal hippocampal infusion of Dkk-1 increased levels of phospho-JNK 5 min after infusion, regardless of whether vehicle or E_2 was infused ICV. (B) Synaptophysin levels were decreased 5 min after DH infusion of Dkk-1. (C) Levels of phospho-PKC were increased 5 min after ICV infusion of E_2, regardless of whether vehicle or Dkk-1 was infused into the DH. Each bar represents the mean ± SEM percent change from vehicle (*$p < 0.05$).

treatment ($F_{(3,28)} = 5.25$, $p < 0.01$, $\eta^2 = 0.36$; Fig. 4B), such that infusion of Dkk-1 + vehicle significantly decreased synaptophysin levels relative to vehicle 45 min post-infusion ($p = 0.0005$). E_2 appeared to mitigate this decrease, as levels in the Dkk-1 + E_2 group did not differ from vehicle ($p = 0.1551$). Phosphorylated PKC protein expression was significantly increased 5 min following E_2 infusion ($F_{(3,18)} = 3.38$, $p < 0.05$; $\eta^2 = 0.36$, Fig. 4C), an effect which was not blocked by Dkk-1 ($p = 0.0142$). These data indicate that Dkk-1 may interface with Wnt/JNK signaling in the hippocampus, but not with Wnt/Calcium signaling.

4. Discussion

Previous work from our laboratory and others has demonstrated that Wnt/β-catenin signaling plays a critical role in memory processes among male mice (Fortress and Frick, 2016; Fortress et al., 2013b; Maguschak and Ressler, 2011). Moreover, numerous reports have revealed that the ability of E_2 to enhance memory consolidation in OVXed mice depends on rapid activation of cell signaling within the DH (Fernandez et al., 2008; Fan et al., 2010; Lewis et al., 2008; Boulware et al., 2013; Fortress et al., 2013a, 2013b). 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 antagonist Dkk-1 prevents E_2-mediated enhancement of object recognition and spatial memory consolidation in OVXed mice. Next, we showed that this 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., 2013b). Our previous work involved DH infusion of a 10-fold higher dose (50 ng/hemisphere) of Dkk-1 than the current study. Here, we found that even a 10 ng/hemisphere dose of Dkk-1 impaired object recognition memory consolidation in females. Therefore, we used a lower dose of Dkk-1 (5 ng/hemisphere) with no effects on memory consolidation on its own to determine whether Dkk-1 interferes with the memory-enhancing effects of E_2. As predicted, Dkk-1 prevented E_2 from enhancing both object recognition and object placement memory consolidation. This result is consistent with data demonstrating that ICV infusion of Dkk-1 reverses the neuroprotective effects of E_2 in the hippocampus of female mice after global cerebral ischemia (Scott and Brann, 2013), and extends these findings to show a novel role for Dkk-1 in preventing E_2-induced memory enhancement among non-brain injured females.

Because Dkk-1 is a known antagonist of Wnt/β-catenin signaling, we hypothesized that it might block the memory-enhancing effects of E_2 by turning "off" Wnt/β-catenin signaling, and thus preventing E_2 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 E_2-mediated increase in these proteins, suggesting that Dkk-1 may actually block E_2-induced memory consolidation through alternative, non-canonical pathways, and that E_2 may not robustly activate Wnt/β-catenin signaling as expected. Indeed, the lack of E_2-mediated increases in Wnt/β-catenin signaling proteins, with the exception of phosphorylated

GSK3β, suggests that E_2 may not enhance memory through direct activation of Wnt/β-catenin signaling. Our data describing an E_2-mediated increase in phosphorylated GSK3β protein are consistent with literature demonstrating a role for E_2 in increasing activation of GSK3β in female rodents (Cardona-Gomez, 2004; Varea et al., 2009). However, GSK3β may interface with additional cell-signaling pathways that are involved in E_2-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 and is predicted to interact with many more (Linding et al., 2007; Sutherland, 2011). 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, E_2-mediated increases in phosphorylated GSK3β may occur independently of Wnt/β-catenin signaling activity.

The modest 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., 2013b), are likely due to our use of a substantially lower, behaviorally sub-effective dose of Dkk-1 in this study. Our previous study was designed to examine possible memory-impairing effects of Dkk-1, and used doses of 50, 100, and 200 ng/hemisphere based on a previous report that bilateral basolateral amygdala infusion of 100 ng/hemisphere impairs fear conditioning in male mice (Maguschak and Ressler, 2011). However, lower doses were necessary for the present study to demonstrate that Dkk-1 specifically interfered with E_2-induced memory enhancement. That is, if a dose of Dkk-1 that impairs memory on its own blocked the memory-enhancing effects of E_2, then it would be unclear if this was due to the memory-impairing effects of Dkk-1 or an interaction between Dkk-1 and E_2. Because we were interested in this potential interaction, the use of a dose of Dkk-1 with no effects on memory itself was necessary. However, because this lower dose had no discernable effect on memory, it may have been too low to produce a measurable increase in Wnt/β-catenin signaling. Our previous study also found that object learning itself triggers Wnt/β-catenin signaling (Fortress et al., 2013b), indicating that object training alone is sufficient to drive changes in protein expression. Tissues in the present study were collected only after drug infusion, not after object training plus drug infusion and thus, the lack of object training immediately prior to drug infusion and tissue collection could have influenced our results. However, our previous study found significant effects of Dkk-1 on Wnt/β-catenin signaling in the absence of object training (Fortress et al., 2013b), albeit with a higher Dkk-1 dose, so we find this explanation somewhat less likely than differences in Dkk-1 dose. An additional possibility is that we examined time points in the current study at which 5 ng/hemisphere Dkk-1 does not exert its effects most strongly. We examined changes in protein expression 5 min after infusion based on our previous work showing that E_2 activates numerous other kinases within 5 min (e.g., Fernandez et al., 2008; Fortress et al., 2013a, 2013b), but were surprised to find limited effects on Wnt/β-catenin signaling at this time point. Inclusion of a 45 min time point was guided by data from our laboratory and others showing that the effects of E_2 on GSK3β and β-catenin activity occur largely within a 1 h window (Cardona-Gomez et al., 2004; Fortress et al., 2013b). Examining more extended time points in future work may aid in characterizing the interactions among E_2, 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 E_2. Importantly, we found no effects of Dkk-1 infusion or E_2 infusion on levels of β-catenin, 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 E_2 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 (Endo et al., 2008; Lee et al., 2004; Chae and Bothwell, 2018; Rosso et al., 2005). Thus, we examined 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 no published studies to the best of our knowledge 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 examined 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).

The resulting data suggest that DH infusion of Dkk-1 increased levels of phosphorylated p46 JNK protein in the DH, whereas ICV infusion of E_2 had no effect on activation of this protein. Furthermore, DH infusion of Dkk-1 decreased synaptophysin protein levels, an effect mitigated by ICV infusion of E_2. Combined, these data suggest that Dkk-1 may exert non-canonical effects in the DH through Wnt/JNK signaling. These data are consistent with other research demonstrating a β-catenin-independent, and JNK-dependent, role for Dkk-1 in other biological processes. For example, in human mesothelioma cell lines deficient in β-catenin, Dkk-1 acts as a tumor suppressor, indicating that Dkk-1 can function 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 my 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., 2017Kühl et al., 2000; McQuate et al., 2017). Here, E_2 increased phosphorylated PKC protein, but Dkk-1 did not mitigate this increase. E_2 may increase levels of phosphorylated PKC independent of Wnt/Calcium signaling activity; indeed, E_2-induced increases in phosphorylated PKC are well documented (Alzamora and Harvey, 2008). Given that infusion of Dkk-1 into the DH did 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 consider that Dkk-1 may not interact with Wnt/β-catenin signaling as specifically as

previously thought. 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 E_2-induced enhancement of memory consolidation via activation of JNK signaling. Recent data from our laboratory demonstrate that the membrane estrogen receptor, G-protein-coupled 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 interplay between Dkk-1 and E_2 may depend on interactions between Dkk-1 other estrogen receptors in the hippocampus, such as ERα or ERβ, rather than GPER. Indeed, the memory-enhancing effects of E_2 appear to activate cell-signaling cascades independent of GPER, given that GPER activation within the DH is not necessary for E_2 to enhance memory (Kim et al., 2016). Future work should investigate the potential estrogen receptor mechanisms mediating the interaction between E_2 and Dkk-1.

In conclusion, the present study demonstrates that Dkk-1 infusion into the DH blocks the effects of E_2 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 insights 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. Importantly, these data suggest a critical role for Wnt signaling in the memory-enhancing effects of E_2, thereby shedding new light on the cell-signaling mechanisms through which E_2 influences memory formation.

Acknowledgements

This work was supported by National Institutes of Health grant R01MH107886 to KMF, a Sex and Gender in Alzheimer's grant (SAGA-17-419092) from the Alzheimer's Association to KMF, the UWM College of Letters and Science, the UWM Office of Undergraduate Research, and Career Development Award Number IK2 BX-003196 from the Biomedical Laboratory Research & Development Service of the VA Office of Research and Development to AMF.

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