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17β-estradiol activation of dorsal hippocampal TrkB is independent of increased mature BDNF expression and is required for enhanced memory consolidation in female mice

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ABSTRACT

The potent estrogen 17β-estradiol (E_2) is known to enhance hippocampal memory and plasticity, however the molecular mechanisms underlying these effects remain unclear. Brain derived neurotrophic factor (BDNF) and its receptor tropomyosin receptor kinase B (TrkB) are regulated by E_2, but the potential mechanistic roles of neurotrophic signaling in E_2-induced enhancement of memory are not well understood. Here, we examined the effects of hippocampal TrkB signaling on E_2-induced enhancement of memory consolidation in the object placement and recognition tasks. Bilateral infusion of the TrkB antagonist ANA-12 into the dorsal hippocampus of ovariectomized female mice blocked E_2-induced enhancement of memory consolidation, supporting a role for TrkB-mediated signaling in estrogenic regulation of memory. Although dorsal hippocampal E_2 infusion increased levels of phospho-TrkB and mature BDNF (mBDNF) in the dorsal hippocampus within 4–6 h, E_2-induced increases in hippocampal mBDNF expression were not required for hippocampal TrkB activation and were not inhibited by TrkB antagonism. Thus, E_2 regulates TrkB signaling to facilitate memory consolidation in a manner independent of mBDNF expression. Together these results provide the first direct evidence that E_2 modulation of hippocampal TrkB signaling is required for its beneficial effects on memory consolidation and provide additional characterization of the ways in which TrkB/BDNF signaling is regulated by E_2 in the hippocampus.

1. Introduction

17β-estradiol (E_2), a potent estrogen, powerfully modulates hippocampal plasticity and memory. E_2 influences the structure and function of hippocampal pyramidal neurons, increasing dendritic spine density and synapse number, thereby leading to potentiated excitatory neurotransmission (Luine and Frankfurt, 2013; Woolley, 1998). These changes are associated with improved performance on hippocampal-dependent memory tasks, including object placement and object recognition tasks (Jacome et al., 2016; Kim et al., 2019; Phan et al., 2012). Although progress has been made in identifying molecular mechanisms that mediate effects of E_2 in the hippocampus (Boulware et al., 2013; Fernandez et al., 2008; Fortress et al., 2013; Kramár et al., 2013; Phan et al., 2012), the field still lacks a comprehensive understanding of the pathways critical for E_2-induced memory enhancement. In particular, accumulating research has suggested that BDNF/TrkB signaling may play a role, but few studies have directly tested this hypothesis.

BDNF is a member of the neurotrophin family of small, secreted peptides and is critically involved in hippocampal synaptic plasticity. Activity-dependent synthesis and release of BDNF drives long-term potentiation, protein synthesis, and structural remodeling in hippocampal synapses (Leal et al., 2015; Park and Poo, 2013) and is required for hippocampal memory (Bekinschtein et al., 2007; Heldt et al., 2007; Lee et al., 2004). BDNF exerts its trophic effects via the receptor TrkB, a tyrosine kinase receptor that activates multiple intracellular signaling pathways involved in neuroplasticity, including ERK, PI3K, and PLC (Park and Poo, 2013; Sasi et al., 2017). Interestingly, activation of these same pathways is also a hallmark of rapid, membrane-initiated E_2 signaling. E_2 treatment rapidly activates hippocampal ERK (Fernandez et al., 2008; Kuroki et al., 2000; Wu et al., 2005), PI3K/Akt (Fan et al., 2010; Hasegawa et al., 2015; Yang et al., 2010), and PLC signaling (Maruyama et al., 2013), and both ERK and PI3K signaling in the dorsal hippocampus are necessary for E_2 to enhance memory in ovariectomized (OVX) mice (Fan et al., 2010; Fernandez et al., 2008; Fortress et al., 2013). The notable overlap in the mechanisms through which E_2 and

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BDNF/TrkB influence hippocampal plasticity and memory has led many to question whether BDNF and TrkB are mediators of rapid E_2 action in this region (Luine and Frankfurt, 2013; Scharfman and MacLusky, 2005; Srivastava et al., 2013).

E_2 has been found to activate both BDNF and TrkB signaling in the rodent hippocampus, which lends considerable support to this hypothesis. E_2 can regulate BDNF on multiple levels, including increasing gene expression through classical genomic activity at an ERE-like site on the *Bdnf* gene (Sohrabji et al., 1995) and epigenetic modification of *Bdnf* (Fortress et al., 2014), and by stimulation of BDNF release (Briz et al., 2015; Sato et al., 2007). OVX of female rodents dramatically reduces BDNF levels in the hippocampus, particularly in the CA3 and dentate gyrus regions, and E_2 replacement restores this expression (Berchtold et al., 2001; Liu et al., 2001; Pan et al., 2010; Scharfman et al., 2007; Singh et al., 1995). E_2 also regulates TrkB activity, as measured by increases in TrkB phosphorylation. In gonadally intact female mice, levels of phospho-TrkB in the hippocampus are greatest during proestrus (Spencer et al., 2008; Spencer-Segal et al., 2011), the phase of the estrous cycle with the highest circulating levels of E_2, and exogenous E_2 treatment increases phospho-TrkB in both hippocampal slices (Kramár et al., 2013; Wang et al., 2016) and the hippocampus of OVX mice (Spencer-Segal et al., 2012). Whether E_2-induced increases in hippocampal TrkB activation are a direct result of enhanced BDNF expression or activity is unclear, as some studies suggest that E_2 can activate TrkB in a BDNF-independent manner (Spencer et al., 2008; Wang et al., 2016).

Despite clear connections between E_2 and BDNF/TrkB in the hippocampus, few studies have directly examined whether BDNF or TrkB signaling play a mechanistic role in the effects of E_2 in this region. In hippocampal culture, manipulation of BDNF or TrkB signaling inhibits E_2-induced dendritic spine formation (Murphy et al., 1998; Sato et al., 2007), and in hippocampal slices from intact female rats, a TrkB inhibitor reduces CA3 hyperexcitability that occurs during proestrus (Scharfman et al., 2003). More recently, it has been shown that TrkB is required for E_2 improvement of spatial memory. In OVX rats, systemic injection of the TrkB antagonist ANA-12 blocked the memory enhancing effects of long-term E_2 replacement on an object placement task (Bohm-Levine et al., 2020). Previous work from our lab has found that a single infusion of E_2 into the dorsal hippocampus (DH) given immediately after training improves spatial and object recognition memory consolidation through acute activation of cell-signaling cascades in the DH during the 1–3 h consolidation period (Boulware et al., 2013; Fan et al., 2010; Fernandez et al., 2008; Fortress et al., 2014, 2013). Whether rapid local activation of TrkB in the DH underlies E_2 enhancement of hippocampal memory consolidation remains unknown. Here, we used hippocampal-dependent object memory tasks to determine if TrkB in the DH is required for acute E_2 infusion to enhance object recognition and spatial memory consolidation. OVX mice received bilateral DH infusions of ANA-12 and intracerebroventricular (ICV) infusion of E_2 immediately following training on object placement or recognition tasks. The effects of E_2 and ANA-12 on TrkB activation and BDNF expression in the DH were also examined. Our findings suggest that TrkB is a critical mediator of the enhancing effects of E_2 on memory consolidation and that E_2-induced TrkB activation is independent of changes to hippocampal BDNF expression.

2. Methods

2.1. Subjects

All subjects were female C57BL/6 mice obtained from Taconic Biosciences at 9 weeks of age. Mice were kept on a 12:12 light-dark cycle with food and water ad libitum and all procedures were conducted between 9:00 A.M. and 6:00 P.M. Mice were housed in groups of up to 5 prior to surgery, after which they were singly housed. All procedures followed the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Milwaukee.

2.2. Surgeries

At 10 weeks of age, mice underwent bilaterally OVX and were implanted with an indwelling guide cannula targeting the dorsal hippocampus (DH) as described previously (Boulware et al., 2013; Fortress et al., 2013). Mice were placed into a stereotaxic apparatus and anesthetized with 5% isoflurane in oxygen, then maintained at 2–3% isoflurane throughout the surgery. Bilateral OVX was performed first, and then guide cannulae (C232G; 22 gauge; Plastics One) were positioned at coordinates for the DH alone (−1.7 mm AP, +/- 1.5 mm ML, −2.3 mm DV) or the DH and dorsal third ventricle (intracerebroventricular (ICV); −0.9 mm AP, 0.0 mm ML, −0.2.3 mm DV). Cannulae were implanted and fixed with dental cement, which also served to close the wound. Dummy cannulae (C232DC, Plastics One) were inserted to keep the guide cannulae clear throughout experiments. Cannula placements were verified visually during tissue dissection. Following surgery, mice were given a minimum of one week to recover before beginning behavioral testing.

2.3. Drugs and infusions

For intracranial infusions, mice were gently restrained, dummy cannulae were removed, and an infusion cannula (C313l; DH: 28 gauge, extending 0.8 mm beyond the 1.5 mm guide; ICV: 28 gauge, extending 1.0 mm beyond the 1.8 mm guide; Plastics One) attached to PE50 polyethylene tubing mounted on a 10 μl Hamilton syringe was inserted. Infusions were controlled by a microinfusion pump (KDS Legato 180, KD Scientific) and administered at a rate of 0.5 μl/min into the DH or 1 μl/2 min into the dorsal third ventricle as described previously (Boulware et al., 2013; Fortress et al., 2013). The infusion cannula was left in place for 1 min following infusion to allow for drug diffusion. In triple infusion experiments using both ANA-12 and E_2 infusion, ANA-12 was first infused bilaterally into the DH, followed immediately by E_2 infusion into the dorsal third ventricle. This triple infusion protocol was used to prevent tissue damage from multiple infusions into the DH in rapid succession (Boulware et al., 2013; Fernandez et al., 2008; Fortress et al., 2013; Zhao et al., 2010).

The TrkB inhibitor, ANA-12 (Tocris Bioscience), was dissolved in 100% DMSO for a stock solution of 4 μg/μl and stored at − 20 °C. On the day of the experiment, dilutions at concentrations of 1 and 2 μg/μl in 80% DMSO were prepared. ANA-12 was infused into the DH at doses of 0.5, 1, or 2 μg/ hemisphere with 80% DMSO serving as the vehicle control. Cyclodextrin-encapsulated E_2 (Sigma-Aldrich) was dissolved in 0.9% sterile saline at a concentration of 10 μg/μl and infused at a dose of 5 μg/hemisphere into the DH or 10 μg ICV. For the vehicle solution, 2-hydroxypropyl-β-cyclodextrin (HBC; Sigma-Aldrich) was dissolved in 0.9% sterile saline at the same concentration as the encapsulated E_2 solution.

2.4. Memory testing

Object recognition (OR) and object placement (OP) tasks were used to measure object recognition and spatial memory as described previously (Boulware et al., 2013; Fernandez et al., 2008; Fortress et al., 2013; Kim et al., 2016). Because all mice completed both tasks, the order of training was counterbalanced within each group and a minimum of two weeks separated training on each task to minimize the potential influences of test order or previous testing on the results (Fig. 1A). Prior to the start of behavioral training, mice were briefly handled for three days and then habituated to the empty testing arena for 5 min on each of two consecutive days. To habituate mice to objects, a Lego Duplo block was placed in each home cage on the second day of handling and remained in the cage until behavioral training began. On the day of behavioral training, mice were briefly rehabituated to the empty arena

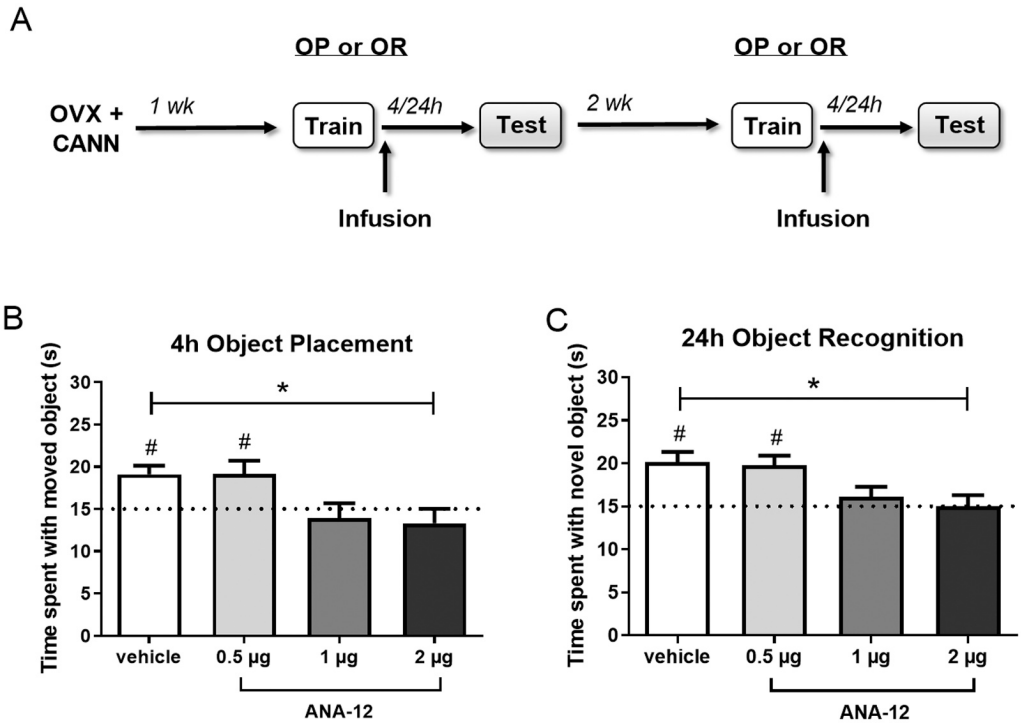

Fig. 1. TrkB antagonism with 1 µg or 2 µg ANA-12, but not 0.5 µg ANA-12, blocks consolidation of object placement and object recognition memory. OVX female mice ($n = 8$–9/group) received bilateral DH infusion of vehicle or ANA-12 (0.5, 1, or 2 µg/hemisphere) immediately following object training (A). In an object placement test 4 h later (B) or an object recognition test 24 h later (C), mice receiving vehicle or 0.5 µg ANA-12 spent significantly more time with the moved or novel object than chance (dotted line at 15 s, #$p < 0.05$ relative to chance), whereas mice receiving 1 or 2 µg ANA-12 did not, suggesting impaired memory consolidation. Mice treated with 2 µg ANA-12 also spent significantly less time with the moved or novel object compared to vehicle treated animals (*$p < 0.05$). Error bars indicate mean ± SEM.

for 1 min, removed, and then placed back into the arena with two identical objects placed near the upper right and left corners. Mice were allowed to explore the objects until they reached a criterion of 30 s of exploration time or until 20 min had elapsed. Exploration was manually scored with ANY-maze tracking software (Stoelting) and was counted when the mouse was immediately adjacent to an object with its front paws and/or nose directed at or touching the object. Mice that successfully reached the 30 s criterion were immediately given drug infusions to target the memory consolidation period and then returned to their home cages.

In the testing trial, one of the objects was either moved to a lower corner of the testing arena (OP) or replaced with a novel object (OR). Because mice inherently prefer novelty, those with intact memory for the training objects should spend more time than chance (15 s) exploring the moved or novel objects. Memory was tested at timepoints previously established by our lab as ideal for observing either impairment or facilitation of memory consolidation (Boulware et al., 2013; Fortress et al., 2013; Kim et al., 2016). That is, to discern possible inhibitory effects of ANA-12 on memory, mice were tested using timepoints at which control mice demonstrate intact memory for the training objects (4 hr delay for OP, 24 hr delay for OR). To observe potential facilitatory effects of E_2, mice were tested using timepoints at which control mice do not demonstrate intact memory (24 hr delay for OP, 48 hr delay for OR). As in training, mice were allowed to explore the objects during testing until they reached the 30-sec exploration criterion or until 20 min had elapsed. They were then returned to their home cages.

2.5. Tissue preparation and Western blot analysis

Because we previously showed that bilateral DH infusion of E_2 increases pro-BDNF and mBDNF protein in the DH of middle-aged OVX mice 4 or 6 h later (Fortress et al., 2014), young behaviorally naïve OVX mice were first used to establish the time course of E_2 effects on TrkB activation and mBDNF protein expression at this age. As in our previous study, tissue was collected 4 and 6 h after infusion. Next, to determine the influence of TrkB inhibition on E_2-induced changes in TrkB and BDNF RNA and protein, the same mice that underwent behavioral testing for interactions between E_2 and TrkB were used for PCR and Western blot analysis of the E_2 +ANA-12 interaction. These mice were euthanized two weeks following the completion of behavioral testing (as indicated in Fig. 4A) to allow for any acute effects of previous infusion and behavioral training to dissipate prior to re-infusion and tissue collection. Tissue was collected 4 h, rather than 6 h, after infusion to capture potentially earlier changes in RNA expression that might precede alterations in protein levels. Mice received intracranial infusions as described above and were then cervically dislocated, decapitated, and brains were removed 4 or 6 h later based on the time course of previously described E_2/BDNF interactions (Fortress et al., 2014). The DH was rapidly dissected on ice and frozen at −80 °C until homogenization. Cannula placements were visually verified during dissection and no missed placements were identified during the study. Western blotting was performed as described previously (Boulware et al., 2013; Fernandez et al., 2008; Fortress et al., 2014, 2013). Tissue was homogenized by sonication in a hypotonic lysis buffer (1:50 w/v) containing PMSF and EDTA-free protease inhibitor cocktail (ThermoFisher Scientific). Total protein concentrations were determined via Bradford assay and then proteins were separated on 4–15% TGX Stain-free precast gels (BioRad) and transferred to PVDF membranes. Membranes were blocked in 5% milk and then incubated with the following primary antibodies at 4 °C overnight: phospho-TrkB (Tyr706, Signalway #11328, 1:1000), TrkB (Cell Signaling Technology #4603, 1:1000), BDNF (Abcam #108319; 1:1000), GAPDH (Cell Signaling Technology #5174, 1:10000). The next day, membranes were washed and incubated with an HRP conjugated secondary antibody (anti-rabbit IgG, Cell Signaling Technology, 1:5000) and developed using Clarity Max enhanced chemiluminescence substrate (Bio-Rad). Blots were imaged using a ChemiDoc MP gel imager (Bio-Rad) and densitometry analysis was performed using ImageLab software (Bio-Rad, Image Lab version 5.2). Lanes were manually defined and bands were detected automatically by the Image Lab software. The Lane Profile tool was used to make minor adjustments to bands and to subtract background. Phospho-TrkB blots were stripped and reprobed for total full-length TrkB (~120 kDa) for normalization. Mature BDNF (15 kDa) was normalized to GAPDH. All normalized proteins were

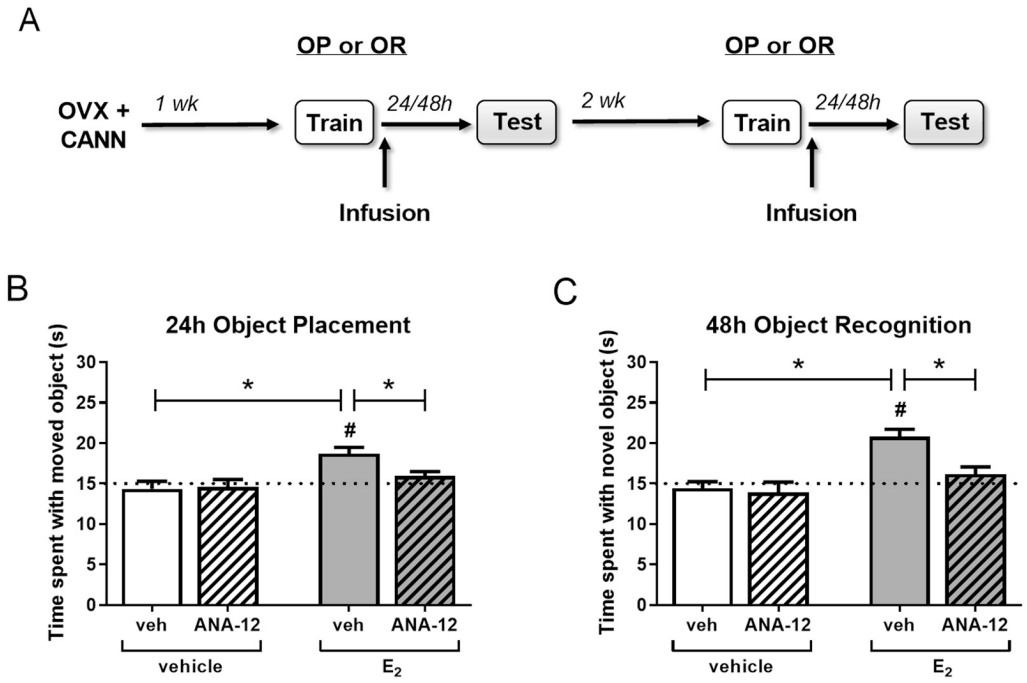

Fig. 2. Estradiol enhancement of memory consolidation depends on hippocampal TrkB. OVX female mice ($n = 10$–16/group) received ICV infusion of vehicle or E_2 (10 μg) and DH infusion of vehicle or ANA-12 (0.5 μg/hemisphere) immediately following object training (A). In an object placement test 24 h later (B) or an object recognition test 48 h later (C), mice infused with E_2 + vehicle spent significantly more time with the moved or novel object than chance (dotted line at 15 s; $\#p < 0.05$), and this effect was eliminated by ANA-12 treatment. Mice infused with E_2 + vehicle also spent significantly more time with the moved or novel object compared to vehicle + vehicle or E_2 + ANA-12 treated animals ($*p < 0.05$). Error bars indicate mean ± SEM.

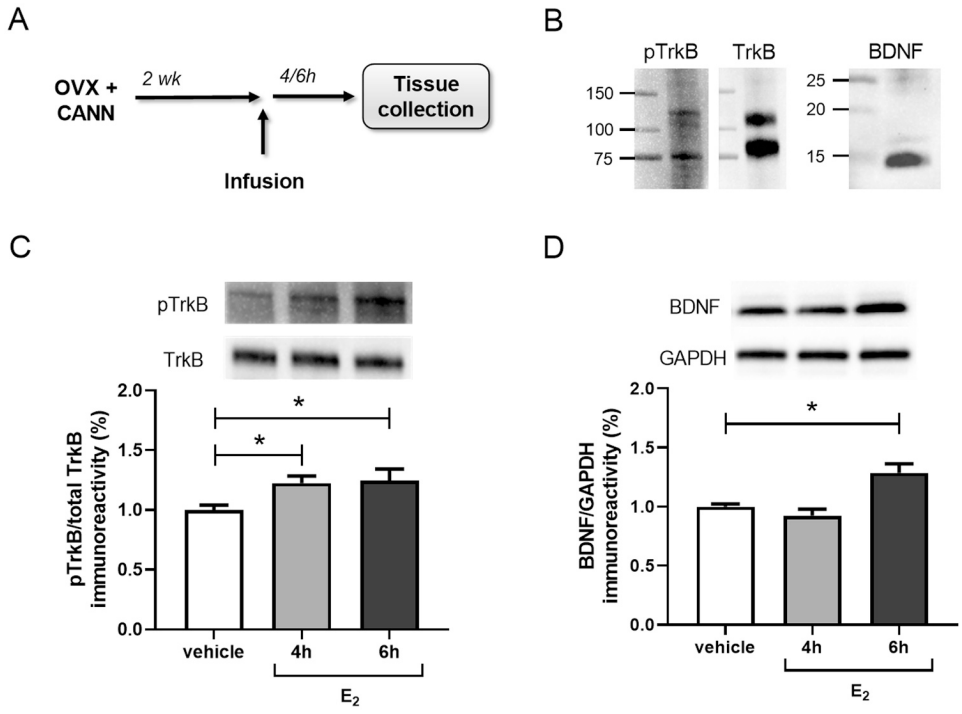

Fig. 3. Estradiol activates hippocampal TrkB prior to increased mBDNF expression. TrkB phosphorylation and mBDNF expression were assessed 4 or 6 h after bilateral DH infusion of vehicle or E_2 (5 μg/hemisphere) in behaviorally naïve OVX female mice (A, $n = 11$–12/group). Full-length (~120 kDa) and truncated (~75 kDa) TrkB and monomeric mBDNF (15 kDa) were detected by Western blot (B). Levels of full-length phospho-TrkB (Tyr706) and full-length total TrkB were used for analysis. E_2 infusion increased levels of phospho-TrkB both 4 and 6 h later (C), but expression of mBDNF was only increased 6 h following E_2 treatment (D). $*p < 0.05$ compared to vehicle control. Error bars indicate mean ± SEM.

expressed as a percentage relative to vehicle control.

2.6. Rt-qPCR

Brain tissue was dissected as described above and stored in RNA*later* (ThermoFisher Scientific) at −20 °C until it was processed. RNA was prepared and quantified as described previously (Fortress et al., 2014; Zhao et al., 2010). Briefly, RNA was extracted using the Qiagen RNeasy Mini kit (Qiagen) following the manufacturer instructions, and cDNA samples were prepared from 1 μg of extracted RNA using the Bio-Rad iScript cDNA Synthesis kit (Bio-Rad). Real-time quantitative PCR (qPCR) was performed using SYBR Green master mix (Bio-Rad) on an Eppendorf Realplex 2 PCR System (Eppendorf). Predesigned and optimized RT^2 qPCR Primer Assays (Qiagen) were used for analysis of total *Bdnf* (#PPM03006C), the TrkB gene *Ntrk2* (#PPM04330A), and *Gapdh* (#PPM02946E; expression control). Results were analyzed using Realplex 2.2 software. Samples were run in triplicate and normalized to corresponding GAPDH values and the ΔΔCT method was used to calculate relative expression of each gene of interest.

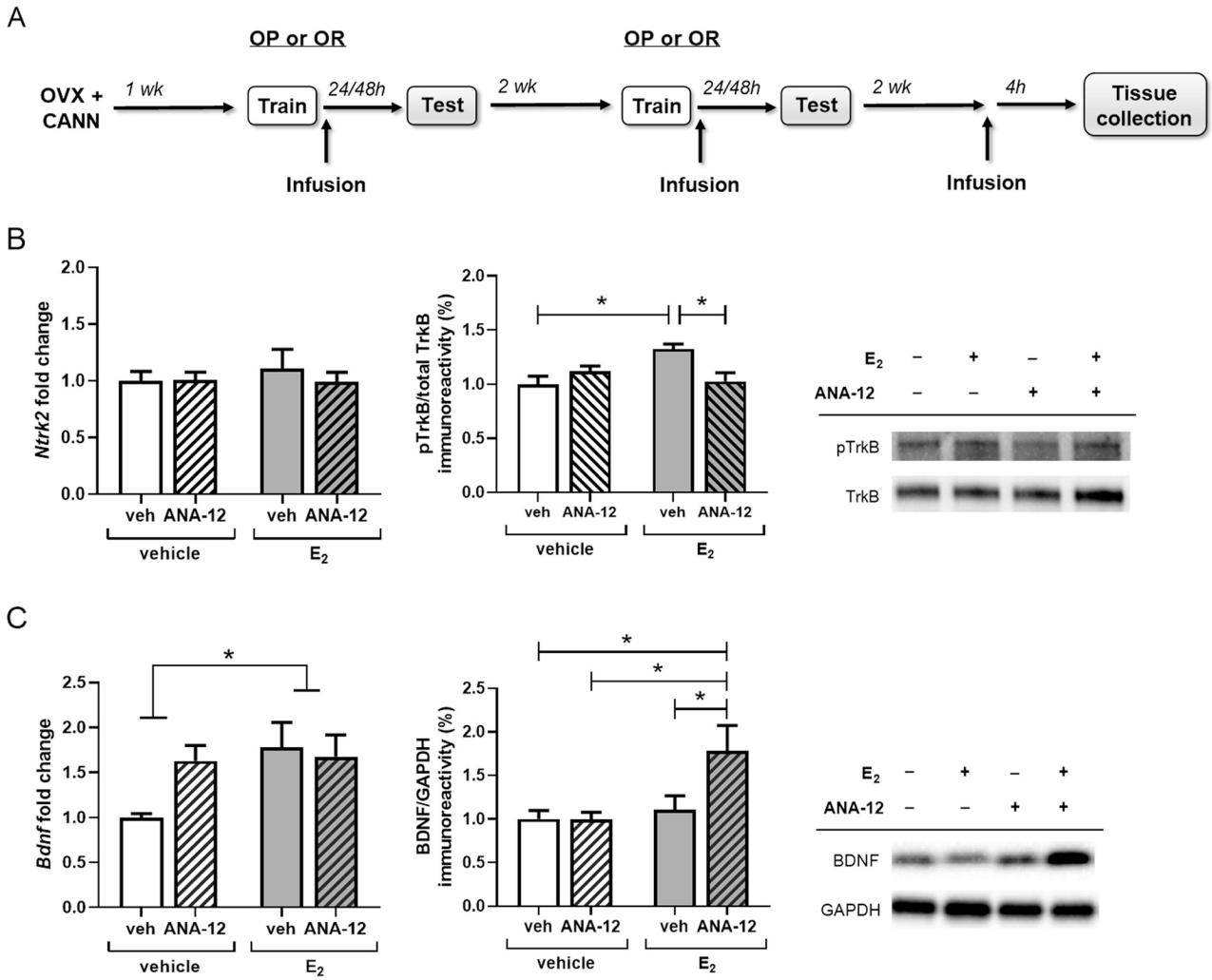

Fig. 4. ANA-12 blocks estradiol-induced TrkB activation and accelerates increased mBDNF expression. TrkB and BDNF expression were assessed 4 h after ICV infusion of vehicle or E_2 (10 μg) and DH infusion of vehicle or ANA-12 (0.5 μg/hemisphere) in OVX female mice (A). Levels of TrkB mRNA were unaffected by E_2 and ANA-12 treatment, but E_2-induced TrkB phosphorylation was blocked by ANA-12 (B, $n = 6$–9/group). Levels of BDNF mRNA were significantly increased by E_2 treatment and E_2 and ANA-12 interacted to increase mBDNF protein at 4 h post-infusion (C, $n = 8$–14/group). *$p < 0.05$ compared to vehicle control. Error bars indicate mean ± SEM.

2.7. Statistical analysis

All statistical analyses were conducted with GraphPad Prism 7. All data were analyzed for outliers, defined by ± 2 standard deviations from the mean, which were removed prior to additional analysis. To assess within-group learning effects, behavioral data were first analyzed with one sample *t*-tests to compare individual group performance to chance (15 s). 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, behavioral data were also analyzed with one- or two-way ANOVAs. For ANA-12 dose-response data, one-way ANOVAs were followed by post hoc Dunnett's test comparing treatment doses to vehicle control. For E_2/ANA-12 behavioral data, two-way ANOVAs (hormone x drug) were followed by planned post hoc comparisons using Fisher's LSD test. Western blot data were analyzed using one- or two-way ANOVAs followed by post hoc Dunnett's test or Tukey's multiple comparison test, respectively. Significance was determined at $p < 0.05$.

3. Results

3.1. ANA-12 impairs hippocampal memory consolidation in a dose dependent manner

Because TrkB activity is involved in hippocampal memory consolidation (Bambah-Mukku et al., 2014; Blank et al., 2016; Minichiello et al., 1999), experiments testing the ability of the TrkB antagonist ANA-12 to inhibit E_2-induced memory enhancement could be confounded by a general inhibitory effect of ANA-12 on memory consolidation. Therefore, we sought to determine a dose of ANA-12 that does not impair memory on its own for use in subsequent experiments with E_2. Immediately after object training, OVX mice received bilateral DH infusion of vehicle or ANA-12 (0.5, 1, or 2 μg/hemisphere, Fig. 1A). To detect possible memory impairing effects of ANA-12, mice were tested 4 hrs later for OP and 24 hrs later for OR, which are time points at which vehicle-treated OVX mice show intact memory (Boulware et al., 2013; Kim et al., 2016). As expected, vehicle treated mice spent significantly more time than chance with the moved ($t_{(7)} = 4.01$, $p = 0.005$, Fig. 1B) and novel ($t_{(7)} = 4.23$, $p = 0.004$, Fig. 1C) objects during testing, indicating intact memory for the training objects. Both the 1 and 2 μg doses of ANA-12 blocked memory formation, as mice in these

groups did not spend significantly more time than chance with the moved (1 μg: $t_{(8)} = 0.61$, $p = 0.56$, 2 μg: $t_{(8)} = 0.98$, $p = 0.36$) or novel objects (1 μg: $t_{(8)} = 1.0$, $p = 0.35$, 2 μg: $t_{(8)} = 0.01$, $p = 0.99$). In contrast, mice receiving 0.5 μg ANA-12 spent significantly more time with the moved ($t_{(7)} = 2.63$, $p = 0.030$) and novel ($t_{(7} = 4.41$, $p = 0.003$) objects, indicating that this dose did not impair memory consolidation in either task. These within-group findings were generally supported by the results of between-group analyses. One-way ANOVAs revealed significant main effects of treatment for both the object placement ($F_{(3, 31)} = 4.15$, $p = 0.014$) and object recognition tasks ($F_{(3, 30)} = 4.64$, $p = 0.009$), with post hoc tests showing that mice treated with 2 μg ANA-12 spent significantly less time with the moved ($p < 0.05$) and novel ($p < 0.05$) objects than vehicle controls. Time to accumulate 30 s of object exploration during testing did not differ among treatment groups for either OP (vehicle: M = 482.9, SEM = 58.9; 0.5 μg: M = 523.1, SEM = 54.6; 1 μg: M = 536.4, SEM = 43.2; 2 μg: M = 567.4, SEM = 54.9) or OR (vehicle: M = 619.8, SEM = 81.9; 0.5 μg: M = 706.4, SEM = 77.9; 1 μg: M = 549.0, SEM = 47.5; 2 μg: M = 691.4, SEM = 79.8), as indicated by one-way ANOVA. Together, these data indicate that ANA-12 dose-dependently blocks memory consolidation, such that 2 μg impairs memory whereas 0.5 μg does not. These findings support an important role for TrkB signaling in mediating memory consolidation among OVX mice and establish a behaviorally sub-effective dose of ANA-12 for use in subsequent studies with E_2.

3.2. ANA-12 blocks estradiol-induced enhancement of memory consolidation

To determine if TrkB signaling is necessary for E_2 to enhance hippocampal memory consolidation, OVX mice received an ICV infusion of vehicle or E_2 (10 μg) and a bilateral DH infusion of vehicle or 0.5 μg/hemisphere ANA-12 immediately following object training (Fig. 2A). To detect possible memory enhancing effects of E_2, mice were tested 24 hrs later for OP and 48 hrs later for OR, which are time points at which vehicle-treated OVX mice exhibit impaired memory (Boulware et al., 2013; Kim et al., 2016). In both tasks, mice treated with vehicle+vehicle or vehicle+ANA-12 did not differ from chance in the time spent with the novel (vehicle+vehicle: $t_{(14)} = 0.47$, $p = 0.65$, vehicle+ANA-12: $t_{(9)} = 1.81$, $p = 0.10$) or moved objects (vehicle+vehicle: $t_{(15)} = 0.86$, $p = 0.41$, vehicle+ANA-12: $t_{(15)} = 1.39$, $p = 0.18$), whereas those treated with E_2 +vehicle demonstrated intact memory by spending significantly more time than chance with the moved ($t_{(12)} = 4.84$, $p = 0.0004$, Fig. 2B) and novel objects ($t_{(9)} = 6.31$, $p = 0.0001$, Fig. 2C). However, mice infused with both E_2+ANA-12 did not spend more time than chance with the moved or novel objects in the object placement ($t_{(9)} = 1.82$, $p = 0.10$) and object recognition tasks ($t_{(15)} = 1.39$, $p = 0.18$), suggesting that ANA-12 blocked the memory-enhancing effects of E_2. Further analysis by two-way (hormone x drug) ANOVA revealed a significant main effect of hormone for object placement ($F_{(1,49)} = 10.81$, $p = 0.002$), and significant main effects of hormone ($F_{(1, 52)} = 17.44$, $p = 0.0001$) and drug ($F_{(1, 52)} = 6.16$, $p = 0.016$), as well as a marginal hormone x drug interaction ($F_{(1, 52)} = 3.90$, $p = 0.054$), for object recognition. Planned post hoc comparisons showed that the E_2+vehicle group spent significantly more time with the moved and novel object compared to the vehicle+vehicle group (OP: $p = 0.0006$, OR: $p = 0.0002$) and E_2 +ANA-12 group (OP: $p = 0.042$, OR: $p = 0.004$) in both tasks. Time to accumulate 30 s of object exploration did not differ among treatment groups in OR when compared by two-way ANOVA (vehicle+vehicle: M = 644.2, SEM = 58.0; E_2+vehicle: M = 717.7, SEM = 68.1; vehicle+ANA-12: M = 626.2, SEM = 41.8; E_2 + ANA-12: M = 644.0, SEM = 58.9). For OP, E_2-treated mice showed a small, but significant, increase in time to complete the task (vehicle+vehicle: M = 558.6, SEM = 44.2; E_2+vehicle: M = 730.9, SEM = 45.8; vehicle+ANA-12: M = 580.7, SEM = 59.7; E_2 + ANA-12: M = 781.4, SEM = 66.7; main effect of hormone: $F_{(1, 49)} = 11.65$, $p = 0.001$). However, this increase did not correlate with observed effects on memory consolidation, as the E_2+vehicle and E_2 +ANA-12 groups did not differ in time to complete the task ($p = 0.54$). This, combined with the lack of effect on accumulation time for OR, suggests an incidental effect on this variable in OP that did not influence memory formation. Together, these results indicate that dorsal hippocampal TrkB activity is necessary for E_2-induced enhancement of object recognition and spatial memory consolidation in OVX mice.

3.3. Estradiol activates hippocampal TrkB signaling prior to increased expression of mBDNF protein

In female mice, E_2 has been shown to increase hippocampal expression of both pro-BDNF and mature BDNF (Fortress et al., 2014; Gibbs, 1999; Scharfman et al., 2007; Singh et al., 1995; Sohrabji et al., 1995). E_2 also increases levels of phosphorylated TrkB (Kramár et al., 2013; Spencer et al., 2008; Spencer-Segal et al., 2012, 2011; Wang et al., 2016), which is an indicator of TrkB activity. Changes in TrkB/BDNF expression and activity are often studied after long-term (>24 hrs) E_2 exposure, however, evidence that E_2 can increase both mature BDNF (mBDNF) protein (Fortress et al., 2014) and TrkB activation (Kramár et al., 2013; Sato et al., 2007) on more rapid timescales has also been reported. Whether TrkB activation influences this more rapid induction of mBDNF protein is unknown. Therefore, we next examined hippocampal TrkB phosphorylation and mBDNF protein expression following acute E_2 treatment. Behaviorally naïve OVX mice were given bilateral DH infusion of vehicle or E_2 (5 μg/hemisphere) and then the dorsal hippocampus was dissected 4 or 6 h later (Fig. 3A), time points at which we have previously observed increased mBDNF expression following DH infusion of E_2 (Fortress et al., 2014). Protein levels of full-length TrkB phosphorylated at Tyr706 (~120 kDa), total full-length TrkB (~120 kDa), and mBDNF (15 kDa) were assessed by Western blot (Fig. 3B). TrkB phosphorylation at Tyr706 positively correlates with tyrosine kinase activity of the receptor and phosphorylation at other sites, including Tyr515 and Tyr816 (Huang and McNamara, 2010; Segal et al., 1996), and can be modulated by OVX and E_2 replacement (Hill et al., 2013, 2012). A significant main effect of E_2 was found for phospho-TrkB expression ($F_{(2, 31)} = 3.90$, $p = 0.031$, Fig. 3C), such that levels of phospho-TrkB were significantly higher than vehicle both 4 and 6 hrs after infusion ($p < 0.05$). A significant main effect of E_2 for mBDNF expression was also observed ($F_{(2, 32)} = 11.49$, $p = 0.0002$, Fig. 3D), but interestingly, mBDNF was increased only at 6 hrs post-treatment ($p < 0.05$). This difference in time courses suggests that E_2-induced TrkB activation precedes increased levels of local mBDNF protein.

3.4. ANA-12 blocks estradiol-induced activation of TrkB and enhances BDNF expression

Finally, we assessed the interactive effect of E_2 and ANA-12 on TrkB/BDNF signaling in the DH. OVX mice used two weeks prior in OR and OP behavioral tasks received ICV infusion of vehicle or E_2 (10 μg) plus DH infusions of vehicle or ANA-12 (0.5 μg/hemisphere) and dorsal hippocampal tissue was collected 4 h later (Fig. 4A). Analysis with two-way ANOVA revealed no effect of hormone ($F_{(1, 23)} = 0.17$, $p = 0.68$), drug ($F_{(1, 23)} = 0.23$, $p = 0.64$), or hormone x drug interaction ($F_{(1, 23)} = 0.33$, $p = 0.57$) on *Ntrk2* mRNA at this timepoint (Fig. 4B). However, E_2 and ANA-12 interacted to influence phospho-TrkB levels (hormone x drug interaction: $F_{(1, 29)} = 10.05$, $p = 0.004$). As seen in our previous experiment (Fortress et al., 2014), mice treated with E_2 +vehicle exhibited significantly increased levels of phospho-TrkB 4 hrs after treatment ($p = 0.010$), however, this effect was blocked by administration of ANA-12 ($p = 0.020$). For *Bdnf* mRNA, the main effect of hormone was significant ($F_{(1,30)} = 4.18$, $p = 0.049$, Fig. 4B), such that transcript levels in E_2-treated groups were increased relative to vehicle-treated groups, regardless of ANA-12 co-administration. For mBDNF protein, a significant main effect of E_2 treatment ($F_{(1,44)} = 6.71$, $p = 0.013$) and a marginal hormone x drug interaction ($F_{(1,44)} = 3.94$, $p = 0.053$) were

observed. Planned post hoc comparisons support that these effects were primarily driven by a combined effect of E_2 +ANA-12 treatment, with E_2 +ANA-12 treated mice expressing significantly higher levels of mBDNF compared to the vehicle+vehicle ($p = 0.011$), vehicle + ANA-12 ($p = 0.017$), and E_2 + vehicle ($p = 0.043$) groups. Together, these results suggest that although ANA-12 treatment blocks E_2-induced TrkB activation, it does not influence E_2 regulation of *Bdnf* expression and may interact with E_2 to accelerate increases in mBDNF protein.

4. Discussion

Previous literature supports a role for TrkB signaling in E_2-induced enhancement of hippocampal memory, however, many of the details of this interaction remain unclear. Here, we examined the ability of E_2 to facilitate hippocampal-dependent memory consolidation among OVX mice in the presence of the TrkB antagonist, ANA-12. We found that ANA-12 infusion into the DH blocked both the memory-enhancing effects of E_2 in OVX mice and E_2-induced phosphorylation of hippocampal TrkB, but had no influence on E_2-induced expression of mBDNF. Further characterization of the relationship between E_2, BDNF expression, and TrkB activation showed that TrkB phosphorylation precedes E_2-induced increases in mBDNF protein. Together, these results demonstrate that activation of hippocampal TrkB signaling is a critical mediator of E_2 enhancement of memory consolidation and provide insight into the complexity of E_2 regulation of BDNF and TrkB.

TrkB activation is known to be important for hippocampal memory (Minichiello et al., 1999) and, in particular, appears to be critical for the process of memory consolidation (Bambah-Mukku et al., 2014; Bekinschtein et al., 2007; Lee et al., 2004). This putative role in consolidation has been studied primarily using inhibitory avoidance, a one-trial associative learning task. In this task, pharmacological blockade of TrkB signaling in the hippocampus during the consolidation period following training impairs memory during testing in male rodents (Bambah-Mukku et al., 2014; Blank et al., 2016; Kim et al., 2012). The present findings extend this work by showing similarly detrimental effects of ANA-12 on memory consolidation in OVX female mice during one-trial spatial and recognition memory tasks. Previous data from OVX rodents has shown that E_2 facilitates consolidation in these tasks within 1–3 h of testing (Fernandez et al., 2008; Frye et al., 2007; Walf et al., 2006). Here, immediate post-training infusion of ANA-12 into the dorsal hippocampus dose-dependently impaired object placement and object recognition memory in OVX mice, demonstrating a necessity for TrkB activation during the memory consolidation period. Together with previous studies, this finding highlights the importance of hippocampal TrkB in consolidation across multiple types of memory and irrespective of E_2.

Next, we examined the interaction of hippocampal TrkB with E_2 in promoting memory consolidation in these tasks. Immediate post-training infusion of E_2 enhanced memory consolidation as previously observed (Boulware et al., 2013; Fernandez et al., 2008; Fortress et al., 2014; Kim et al., 2016). However, in mice that also had a post-training infusion of low dose ANA-12 into the hippocampus, this effect was abolished, demonstrating that E_2 enhancement of memory consolidation on object placement and object recognition tasks depends on hippocampal TrkB activity. These results extend previous work correlating estrogenic modulation of hippocampal memory with BDNF/TrkB activity (Luine and Frankfurt, 2013; Pan et al., 2010; Scharfman et al., 2007) and the recent finding of TrkB's involvement in long-term E_2's enhancement of spatial memory in OVX rats (Bohm-Levine et al., 2020) by demonstrating E_2-induced enhancement of spatial and object recognition memory consolidation in OVX mice relies on dorsal hippocampal TrkB activation. The fact that TrkB is essential for the memory-enhancing effects of E_2 in OVX rats and mice may indicate a generalizable role across species. Moreover, work focused on other brain regions and memory systems suggests that activation of TrkB signaling may be a common mechanism of E_2-induced learning. Systemic ANA-12 treatment blocks E_2-induced facilitation of extinction learning on a cocaine conditioned place preference test in OVX rats, and this behavior is correlated with a TrkB dependent potentiation of infralimbic medial prefrontal cortex neurons by E_2 (Yousuf et al., 2019). Future work examining the mechanisms of E_2 modulation of cognition should consider how E_2 activation of TrkB signaling may work throughout the brain to modulate multiple types of learning and memory.

TrkB contributes to hippocampal plasticity and memory through its activation of multiple downstream signaling pathways, including MEK/ERK, PI3K/Akt, and PLC (Leal et al., 2015). These pathways are also critical for E_2-induced plasticity and memory (Frick, 2015), which is a central reason that TrkB has been hypothesized to mediate the effects of E_2 in the hippocampus. Our findings support a mechanistic role for TrkB activation in E_2-induced enhancement of hippocampal memory consolidation; however, many questions remain about how E_2 activation of TrkB facilitates memory and how TrkB activation can be integrated into our current understanding of the molecular mechanisms driving E_2 effects on hippocampal plasticity and function. Previous studies examining the in vivo and *ex vivo* activation of kinase networks in male and female rodents by acute E_2 treatment have shown that these pathways can be activated very rapidly, within 5–30 min of E_2 exposure (Fan et al., 2010; Fernandez et al., 2008; Hasegawa et al., 2015; Kuroki et al., 2000). This rapid activation has also been shown to depend on other cell surface receptors and channels, such as mGluRs (Boulware et al., 2013, 2005) and L-type calcium channels (Wu et al., 2005). We anticipated that TrkB activation, however, might occur at later time points due to previous findings that hippocampal infusion of E_2 does not increase mBDNF expression until 4–6 h later (Fortress et al., 2014). Here, we observed phosphorylation of TrkB at Tyr706 at both 4 and 6 h following E_2 treatment, indicating an increase in TrkB kinase activity. Although we cannot rule out an earlier phosphorylation of TrkB at this or other sites, there is other evidence that TrkB activation is temporally distinct from other mechanisms of rapid E_2 signaling in the hippocampus. Specifically, systemic E_2 replacement in OVX mice induces early hippocampal Akt activation followed by a delayed increase in TrkB phosphorylation (Spencer-Segal et al., 2012), and in E_2 treated hippocampal slices from male rats, β1-integrin activation that facilitates synaptic signaling precedes activation of TrkB (Wang et al., 2016). It is possible that E_2's influence on hippocampal plasticity and memory consolidation occurs in stages, with an initial rapid kinase activation mediated by one set of mechanisms followed by a TrkB-mediated wave of signaling that contributes to longer term plasticity and consolidation. Indeed, others have found evidence that neuroplasticity resulting from rapid E_2 signaling requires further stimulation for changes to synaptic structure and transmission to persist (Srivastava, 2012). Previous work from our lab has found that hippocampal E_2 infusions induce rapid epigenetic modifications within 30 min that impact BDNF expression 4–6 h later (Fortress et al., 2014), lending further support to the idea that rapid E_2 signaling is necessary for initiating longer term effects that contribute to memory consolidation. Similarly, work on the mechanisms of memory generally supports a role for BDNF signaling in later stages of memory consolidation and maintenance (Bekinschtein et al., 2007). In questioning how E_2-induced TrkB activation contributes to hippocampal synaptic plasticity and memory, it is also worth considering the localization of E_2-induced pTrkB. Among intact female mice, hippocampal pTrkB is expressed primarily in presynaptic terminals, and these levels become elevated during proestrus in stratum radiatum axons (Spencer-Segal et al., 2011). How this change in localization contributes to E_2-induced neuroplasticity remains unclear. To further comprehend how E_2 and TrkB interact to facilitate hippocampal memory consolidation, a more detailed understanding of when and how hippocampal TrkB is activated following E_2 treatment and the specific molecular and cellular consequences of E_2-induced TrkB signaling will be critical questions for future work.

In addition to testing the involvement of TrkB in E_2 enhancement of hippocampal memory consolidation, we also examined the relationships

among E_2, hippocampal TrkB activation, and BDNF expression. E_2 is known to activate hippocampal TrkB (Kramár et al., 2013; Spencer-Segal et al., 2012), but the mechanisms through which this occurs remain unclear. The ability of E_2 to increase levels of hippocampal mBDNF offers one explanation (Murphy et al., 1998; Scharfman et al., 2003), but our finding that E_2-induced increases in hippocampal pTrkB occur at least two hours prior to increased mBDNF suggests that E_2 also regulates TrkB activity through mechanisms beyond a simple increase in ligand expression. Other research has found a similar uncoupling between E_2 regulation of BDNF expression and TrkB activity that may be relevant to our findings. In a study of ER knockout mice, OVX ERα knockouts demonstrate a loss of E_2-induced TrkB activation despite intact E_2 induction of BDNF mRNA (Spencer-Segal et al., 2012). In hippocampal cultures, E_2 increases PSD-95 in a TrkB-dependent manner, but without a correlating increase in mBDNF expression (Sato et al., 2007). Rather, E_2 was found to increase BDNF release from dentate gyrus cells through a membrane-initiated rapid signaling mechanism (Sato et al., 2007). Similarly, TrkB-dependent E_2 stimulation of mammalian target of rapamycin (mTOR) signaling in hippocampal culture is associated with a rapid E_2-induced release of BDNF (Briz et al., 2015). Modulation of BDNF release is one possible route for E_2-induced activation of TrkB in the absence of changes to BDNF levels, however, TrkB activation could also be independent of BDNF. BDNF-independent transactivation of TrkB is a well-established phenomenon that can occur through extracellular zinc release or through intracellular signaling downstream of GPCRs, such as adenosine and endocannabinoid receptors (Nagappan et al., 2008). An interesting point of convergence in both of these mechanisms is the activation of Src-family kinases to tyrosine phosphorylate, and ultimately activate, the TrkB receptor (Nagappan et al., 2008). Recently, evidence of TrkB transactivation downstream of E_2 signaling has been found in the hippocampus. In hippocampal slices from male mice, E_2 increases TrkB phosphorylation within 30 min of treatment and this effect remains intact in the presence of a BDNF scavenger (Wang et al., 2016), suggesting a BDNF-independent means of TrkB activation that appears to involve β1-integrins. Src kinases could also play a role, as E_2 is known to activate Src signaling in the hippocampus (Bi et al., 2000; Wu et al., 2005). Determining which, if any, of these mechanisms contribute to E_2 activation of hippocampal TrkB in vivo should be examined in future studies.

We also found that ICV administration of E_2 had no effect of TrkB transcription 4 h later and that E_2 +ANA-12 treatment blocked TrkB activation but did not block, and may have accelerated, induction of BDNF expression. Previous work on the effects of E_2 on hippocampal TrkB expression indicate mixed results, showing both upregulation (Pan et al., 2010) and no change to TrkB expression (Solum and Handa, 2002; Spencer-Segal et al., 2012) following E_2 treatment. This variability in findings is likely due to experimental differences, including length, dosage, and route of E_2 replacement, as well as the animal models used. Further research will be needed to fully understand how E_2 may regulate TrkB gene expression. The increase in hippocampal mBDNF expression following E_2 treatment is in line with our previous findings demonstrating that DH infusion of E_2 increases mBDNF protein in OVX mice within 6 h (Fortress et al., 2014). That TrkB antagonism doesn't inhibit this increase in expression is not necessarily surprising, as currently known mechanisms of E_2 upregulation of BDNF expression, such as binding at an ERE-like site (Sohrabji et al., 1995) and epigenetic regulation (Fortress et al., 2014), are not thought to involve TrkB activation. The observed interaction between E_2 and ANA-12 to increase mBDNF protein at 4 h, prior to when increases from E_2 alone occur, may be due to a compensatory mechanism that drives ligand upregulation as a result of TrkB antagonism.

In conclusion, this study provides important new insights about the role of TrkB in hippocampal memory consolidation and its interaction with E_2 to mediate memory enhancement. We extend previous work on the function of TrkB in memory consolidation by demonstrating a necessity for TrkB signaling in spatial and object memory consolidation and provide the first evidence that hippocampal TrkB receptors are essential for E_2 enhancement of hippocampal memory consolidation. Although further work must be conducted to elucidate the mechanisms through which E_2 activates hippocampal TrkB, our data also provide evidence that E_2 activation of TrkB occurs independently of E_2-induced changes to mBDNF expression. Together, these findings shed new light on the importance of TrkB in E_2-mediated regulation of hippocampal function and the complex interactions among E_2, TrkB, and BDNF signaling in the hippocampus.

CRediT authorship contribution statement

Karyn M. Frick: Funding acquisition, Conceptualization, Project administration, Supervision, Writing - review & editing. **Kellie S. Gross:** Conceptualization, Data collection, Formal analysis, Supervision, Writing - original draft, Writing - review & editing. **Randie L. Alf:** Data collection, Writing - review & editing. **Tiffany R. Polzin:** Data collection, Writing - review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dr. Karyn M. Frick is a co-founder of, and shareholder in, Estrigenix Therapeutics, Inc., a company which aims to improve women's health by developing safe, clinically proven treatments for the mental and physical effects of menopause. She also serves as the company's Chief Scientific Officer. The other authors have no competing interests to declare.

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