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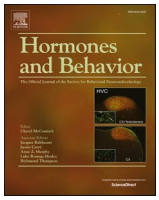

Astrocytic glutamate transport is essential for the memory-enhancing effects of 17β-estradiol in ovariectomized mice

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ABSTRACT

Infusion of 17β-estradiol (E_2) into the dorsal hippocampus (DH) of ovariectomized (OVX) mice enhances memory consolidation, an effect that depends on rapid phosphorylation of extracellular signal-regulated kinase (ERK) and Akt. Astrocytic glutamate transporter 1 (GLT-1) modulates neurotransmission via glutamate uptake from the synaptic cleft. However, little is known about the contribution of DH astrocytes, and astrocytic glutamate transport, to the memory-enhancing effects of E_2. This study was designed to test whether DH astrocytes contribute to estrogenic modulation of memory consolidation by determining the extent to which DH GLT-1 is necessary for E_2 to enhance memory in object recognition and object placement tasks and trigger rapid phosphorylation events in DH astrocytes. OVX female mice were bilaterally cannulated into the DH or the DH and dorsal third ventricle (ICV). Post-training DH infusion of the GLT-1 inhibitor dihydrokainic acid (DHK) dose-dependently impaired memory consolidation in both tasks. Moreover, the memory-enhancing effects of ICV-infused E_2 in each task were blocked by DH DHK infusion. E_2 increased p42 ERK and Akt phosphorylation in DH astrocytes, and these effects were blocked by DHK. Results suggest the necessity of DH GLT-1 activity for object and spatial memory consolidation, and for E_2 to enhance consolidation of these memories and to rapidly activate cell signaling in DH astrocytes. Findings indicate that astrocytic function in the DH of OVX females is necessary for memory formation and is regulated by E_2, and suggest an essential role for DH astrocytic GLT-1 activity in the memory-enhancing effects of E_2.

1. Introduction

The pro-cognitive effects of estrogens on hippocampus-dependent memory in young female rodents are well documented (Frick, 2015; Luine et al., 2018; Luine and Frankfurt, 2020; Taxier et al., 2020). However, much less is known about the cell-type specific mechanisms through which estrogens benefit memory processes. Estrogenic enhancement of memory depends upon the rapid activation of dorsal hippocampal cell-signaling cascades, including extracellular signal regulated kinase/mitogen activated protein kinase (ERK/MAPK) and Akt signaling (Fan et al., 2010; Fernandez et al., 2008). Presumably, these cell-signaling cascades are activated within hippocampal neurons following acute infusion of the potent estrogen 17β-estradiol (E_2) directly into the dorsal hippocampus (DH). However, the presence of estrogen receptors in astrocytes suggests that they may also be a target for estrogenic action and subsequent downstream signaling events (Azcoitia et al., 1999; Chaban et al., 2004). Indeed, although various astrocytic functions seem to be critical for hippocampal memory formation (Ben Menachem-Zidon et al., 2011; Li et al., 2020; Newman et al., 2011; Suzuki et al., 2011; Tian et al., 2019), whether E_2 interacts

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with astrocytes in the hippocampus to facilitate memory consolidation is unknown.

A single astrocyte can ensheath tens of thousands of synapses, positioning itself ideally to perform numerous functions critical for synaptic plasticity including release of gliotransmitters, glutamate synthesis and reuptake, and synthesis and release of trophic factors (Danbolt, 2001; De Pittà et al., 2016). Recent work, conducted largely in male rodents, highlights a facilitatory role for astrocytic activity on memory consolidation in multiple paradigms including inhibitory avoidance (Gao et al., 2016; Suzuki et al., 2011), object recognition memory (Gibbs et al., 2011; Gibbs et al., 2006; Gibbs and Bowser, 2009; Robin et al., 2018; Tian et al., 2019; Vignoli et al., 2016), spatial memory (Adamsky et al., 2018), and contextual fear memory (Adamsky et al., 2018; Kol et al., 2020), indicating that astrocytes are far more active participants in memory formation than originally appreciated.

One critical function of astrocytes linked to their participation in memory processes is the removal of glutamate from the synaptic cleft via glutamate transporter 1 (GLT-1, aka excitatory amino acid transporter 2 or EAAT2), which is expressed primarily in astrocytes (Jensen et al., 2015). GLT-1 is responsible for over 90 % of total glutamate uptake in the brain, making it a major contributor to maintaining glutamate homeostasis and preventing excitotoxicity (Heo et al., 2012; Jensen et al., 2015; Rothstein et al., 1996). GLT-1 is highly expressed by astrocytes in the hippocampus, in which disruption of glutamatergic tone impairs hippocampus-dependent memory and is associated with neurodegenerative diseases (Heo et al., 2012; Jensen et al., 2015; Pajarillo et al., 2019). Genetic knockout of GLT-1 impairs induction of hippocampal long-term potentiation (LTP) in male mice (Katagiri et al., 2001). Inhibition of GLT-1 with dihydrokainic acid (DHK) blocked increased glutamate uptake occurring during chemically-induced late LTP in mouse hippocampal slices (Pita-Almenar et al., 2012). Additionally, hippocampal glutamate clearance increased during late LTP in a GLT-1-dependent manner, suggesting the importance of GLT-1 in dynamically regulating glutamatergic tone (Pita-Almenar et al., 2012). Accordingly, astrocytic clearance of extracellular glutamate via GLT-1 appears to be crucial for memory consolidation. For example, intracerebroventricular (ICV) infusion of DHK impairs novel object recognition memory in male mice (Tian et al., 2019) and spatial memory in male rats (Bechtholt-Gompf et al., 2010). Collectively, this work implicates GLT-1 as a key regulator of memory processes, although the specific involvement of hippocampal GLT-1 in memory formation is unknown.

Astrocytes throughout the brain express all three major estrogen receptors (ERα, ERβ, and GPER), providing binding sites for the most prevalent circulating estrogen, E_2 (Acaz-Fonseca et al., 2014; Arevalo et al., 2010; Azcoitia et al., 1999; Kuo et al., 2010; Rurak et al., 2021), which is synthesized by both neurons and astrocytes (Brann et al., 2022). Moreover, estrogens interact with synapse-associated astrocytes in multiple brain areas. In the hippocampus, astrocyte volume (Klintsova et al., 1995) and levels of the astrocytic protein GFAP fluctuate across the estrous cycle, such that the highest levels of GFAP occur concurrently with high levels of E_2 (Arias et al., 2009). Intracellular calcium concentration in cultured neonatal astrocytes was rapidly increased following bath application of E_2, suggesting that, like in neurons, E_2 initiates rapid intracellular signaling in astrocytes (Chaban et al., 2004; Kuo et al., 2010). Indeed, in cultured midbrain and cortical astrocytes, E_2 rapidly activates ERK and Akt, respectively (Dhandapani et al., 2005; Ivanova et al., 2001). E_2 or estrogen receptor agonists also increase protein and mRNA levels of other astrocytic factors, including GLT-1, in cortical and midbrain astroglial cultures from healthy controls (Lee et al., 2012b; Lee et al., 2012a; Pawlak et al., 2005) and from human patients with Alzheimer's disease (Liang et al., 2002), indicating a potential role for E_2 in modulating hippocampal glutamatergic synaptic plasticity via alterations in glutamate reuptake from the synaptic cleft.

Existing work documenting that E_2 interacts with astrocytes in a manner that may facilitate glutamatergic synaptic plasticity led us to hypothesize that astrocytic GLT-1 in the dorsal hippocampus may be critical for the memory-enhancing effects of E_2. Therefore, the present study was designed to determine whether pharmacological blockade of GLT-1 in the DH would both inhibit object recognition and object placement memory consolidation in ovariectomized female mice and prevent E_2 from enhancing memory consolidation in females. Furthermore, we isolated protein from hippocampal astrocytes to evaluate whether E_2-initiated cell signaling events critical for memory consolidation occur in astrocytes. Our findings suggest that DH GLT-1 activity is necessary in females for both baseline memory formation and for the memory-enhancing effects of E_2, and that E_2 activates ERK and Akt signaling in hippocampal astrocytes in a GLT-1-dependent manner. These data provide the first evidence indicating an essential role for hippocampal astrocytes, and astrocytic GLT-1 function in particular, in mediating both memory formation and the memory-enhancing effects of E_2 in female mice.

2. Methods

2.1. Subjects

Female C57BL/6 mice ($n = 48$ for single drug experiments; $n = 57$ for multiple drug experiments) were obtained from Taconic Biosciences at 8 weeks of age. Mice were maintained on a 12-h light-dark cycle and were given ad libitum access to food and water. Mice were housed in groups of up to five until 24 h before surgery. After surgery, which occurred two weeks prior to the start of behavioral testing, mice remained singly housed for the duration of the experiment. All procedures followed the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee.

2.2. General experimental design

A series of three studies were conducted to examine the roles of GLT-1 on memory consolidation and astrocytic cell signaling. Object recognition (OR) and object placement (OP) tasks were used to assess involvement of GLT-1 in the consolidation of object recognition and spatial memories, respectively. These tasks were selected because memory consolidation in both depends on intact DH function in ovariectomized female mice (Frick, 2015) and because bilateral DH infusion of E_2 consistently enhances memory consolidation in both tasks among ovariectomized mice (Tuscher et al., 2018). We first conducted a dose-response study involving three doses of the GLT-1 inhibitor DHK to identify doses that might impair memory consolidation and those that have no effect on memory on their own. This was necessary to: 1) establish a critical role for hippocampal GLT-1 in mediating OR and OP memory consolidation in females, and 2) determine a dose of GLT-1 that did not impair memory on its own that could be infused with E_2 to determine possible interactions between E_2 and GLT-1. Next, we infused vehicle or DHK into the DH and vehicle or E_2 into the dorsal third ventricle (ICV) to determine the extent to which DHK blocks the beneficial effects of E_2 on memory consolidation in both tasks. In this triple cannulation approach, mice are implanted with bilateral DH cannulae plus a unilateral ICV cannula adjacent to the DH. We have successfully used this approach in several studies to deliver E_2 and inhibitor compounds to the DH without risking tissue damage in the DH due to multiple infusions in succession (Gross et al., 2022; Kim et al., 2016; Taxier et al., 2019). Finally, hippocampi of triple-cannulated mice were collected 5 min after a final infusion to measure effects of E_2 and DHK on ERK and Akt phosphorylation in hippocampal astrocytes.

2.3. Ovariectomy and cannula implantation surgery

At 9 weeks of age, mice underwent a single surgical procedure during which they were first bilaterally ovariectomized (OVXed), and then

implanted with cannulae into the DH or both DH and ICV, as described previously (Gross et al., 2022; Kim et al., 2016; Taxier et al., 2019). For the DHK dose-response experiment, bilateral guide cannulae (Plastics One, Roanoke, VA) were implanted into the DH (C232GC, 22 gauge; −1.7 mm AP, ± 1.5 mm ML, and − 2.3 mm DV). For the DHK-E_2 interaction experiments, triple guide cannulae were implanted into the DH and ICV (C232GC, 22-gauge, −0.9 mm AP, ± 0 ML, and − 2.8 mm DV). Dummy cannulae were inserted to prevent clogging of cannula tracts, and dental cement (Darby Dental) was used to affix the cannulae to the skull and close the wound. Mice were provided a single dose of Rimadyl (1:100; 10 ml/kg) at the start of surgery for analgesia, and one dose per day for two days following surgery for postoperative analgesia. Mice recovered for one week prior to the start of handling for behavioral experiments.

2.4. Drugs and infusions

The GLT-1 inhibitor dihydrokainic acid (DHK, $C_{10}H_{17}NO_4$, Sigma-Aldrich, St. Louis, MO) binds competitively and exclusively to GLT-1 (Arriza et al., 1994). Although low levels of GLT-1 can be found in neurons (Chen et al., 2004), this transporter is highly and primarily expressed in astrocytes in the hippocampus and elsewhere in the brain (Lehre et al., 1995; Rothstein et al., 1994); as such, DHT exhibits a high degree of selectivity for astrocytes and astrocytic GLT-1, and is widely considered a principal blocker of astrocytic glutamate transport in the brain. DHT was dissolved in 0.9 % sterile saline to concentrations of 1, 3.25, or 6 nM based on doses that impacted memory in males in previous work (Bechtholt-Gompf et al., 2010; Tian et al., 2019). For single-drug experiments, 0.5 μl saline vehicle or DHK (1, 3.25, or 6 nM) was infused bilaterally into the DH at a rate of 0.5 μl/min immediately after training in the OR and OP tasks. For studies in which both E_2 and DHK were infused, cyclodextrin-encapsulated E_2 was dissolved in 0.9 % sterile saline to a concentration of 10 μg/μl and infused at a dose of 10 μg as per our lab's previous studies (Fortress et al., 2013; Gross et al., 2022; Kim et al., 2016; Taxier et al., 2019). The vehicle, 2-hydroxypropyl-ß-cyclodextrin (HBC; Sigma-Aldrich), was dissolved in 0.9 % sterile saline to the same concentration of cyclodextrin present in the cyclodextrin-encapsulated E_2 solution. For double-drug experiments, a volume of 0.5 μl saline vehicle or 1 nM DHK was infused bilaterally into the DH as described above, and immediately followed by an ICV infusion of 1 μl HBC or E_2 at a rate of 0.5 μl/min for 2 min.

2.5. Object recognition and object placement

OR and OP memory tasks were conducted as described previously (Boulware et al., 2013; Taxier et al., 2019). Briefly, mice were handled for 5 min/day for 3 days prior to the start of behavioral training. Next, mice were habituated to the empty arena for 5 min/day for 2 days. The following day, mice were placed into a dimly lit open field in which two identical objects were placed near the northwest and northeast corners. Mice explored the objects until they reached a criterion of 30 s of exploration time, or until 20 min had elapsed. Mice that did not acquire 30 s of exploration time within 20 min were excluded. During OR testing, a novel object was substituted for one training object and mice again acquired 30 s of object exploration within 20 min. During OP testing, one training object was moved to the southwest or southeast corner of the arena where mice again had 20 min to accumulate 30 s of object exploration. Because mice prefer to explore novelty, intact memory of the training objects was demonstrated if mice spent significantly more time than chance (15 s) with the novel or moved object. For the DHK dose-response experiment, OR and OP testing occurred at short delays of 24 h (OR) and 4 h (OP). For the DHK-E_2 interaction experiment, OR and OP testing occurred at longer delays of 48 h (OR) and 24 h (OP). Because vehicle-treated mice exhibit intact memory following a 24 h (OR) and 4 h (OP) delay (Taxier et al., 2019), we used these short delays to determine memory impairing and non-impairing doses of DHK. At the longer 48 h and 24 h delays, vehicle-treated mice exhibit impaired memory for a previously-seen object or location, and we consistently find that DH or ICV E_2 infusion enhances memory consolidation in both tasks (e.g., Gross et al., 2022; Kim et al., 2016; Taxier et al., 2019). Therefore, the 48 h (OR) and 24 h (OP) delays allowed us to assess whether a non memory-impairing dose of DHK could interfere with the memory-enhancing effects of E_2.

2.6. Magnetic activated cell sorting (MACS)

Astrocytes in the DH were isolated using MACS. Collection of DH tissue for MACS occurred two weeks following the completion of behavioral testing in the DHK-E_2 interaction experiment. Behaviorally-tested mice were reinfused with their original treatments of vehicle or DHK into the DH, and vehicle or E_2 ICV, and were cervically dislocated and decapitated 5 min later. Whole hippocampus was collected bilaterally and stored at 4 °C in MACS Tissue Storage Solution (Miltenyi Biotec). For optimal protein yield, hippocampi were pooled from 3 to 4 mice, resulting in 4 pools/treatment condition. MACS was performed following the manufacturer's instructions in combination with a modified published protocol (Chisholm et al., 2015). Briefly, hippocampi were removed from MACS Tissue Storage Solution and minced finely with a razor blade before being gently homogenized in proprietary enzymes and buffers (Miltenyi Adult Brain Dissociation Kit, Miltenyi Biotec) while heating in a water bath at 37 °C. Homogenates were strained into 50 ml conical tubes using 70 μM MACS smart strainers. Then, homogenates underwent debris and red blood cell removal, followed by a brief incubation in FcR blocking reagent to prevent nonspecific binding of B cells, monocytes, and macrophages as per kit instructions. Astrocytes were then positively selected using ACSA-2 magnetic beads (Miltenyi Biotec). The ACSA-2-labeled cell suspension was then added to LS columns, and the positive fraction collected and stored at −80 °C for subsequent Western blotting.

2.7. Western blot

Hypotonic lysis buffer (150 μl) was added to astrocyte-positive hippocampal fractions from MACS, and protein content was assayed using the Bradford protein assay. Proteins (10 μg/well) were electrophoresed on 4–15 % TGX precast gels (Bio-Rad) and transferred to PVDF membranes (Bio-Rad). Western blots were blocked with 5 % skim milk prior to incubation with primary antibodies (phospho ERK, phospho-Akt, the astrocytic protein GFAP, the neuronal protein NeuN, and the microglial protein Iba1) overnight at 4 °C. The following day, blots were incubated for 1 h at room temperature with secondary antibody (1:5000, Cell Signaling Technology) and developed using ECL Max substrate (Bio Rad). Signal was detected with a ChemiDoc MP gel imager (Bio Rad) and densitometry was performed using ImageLab software (BioRad). Blots were then stripped and reprobed for total protein (total ERK, total Akt), or β-actin. Phosphorylated protein content was normalized to total protein content, and GFAP, NeuN, and Iba1 were normalized to β-actin. Data were represented as percent immunoreactivity relative to vehicle-treated controls.

2.8. Statistical analyses

Statistical analyses were performed using GraphPad Prism 9 software (La Jolla, Ca). As described previously (Gross et al., 2022; Kim et al., 2016; Taxier et al., 2019), behavioral data were first analyzed using one-sample *t*-tests to determine whether each group spent more time with the novel or moved object relative to chance (15 s, which represents equal exploration of each object). This within-group comparison to a fixed chance value is essential to determine the degree to which learning occurred within each group (Gervais et al., 2013; Gresack and Frick, 2006; Pereira et al., 2014). Next, analyses of variance (ANOVAs) were used to evaluate between-group treatment effects. The DHK dose-

response experiment used one-way ANOVAs with treatment as the dependent variable. The DHK-E_2 interaction experiment used two-way ANOVAs with DHK or E_2 treatment as the dependent variables. As indicated above, mice that did not explore up to the 30 s criterion were excluded from behavioral data analyses. Western blotting data were analyzed using similar one- and two-way ANOVAs. Significant interactions for all ANOVAs were followed by Tukey's post hoc analyses, with significance determined at the $p < 0.05$ level. Effect sizes were calculated using η_p^2 for ANOVAs and Cohen's d for pair-wise comparisons (Lakens, 2013).

3. Results

3.1. GLT-1 is necessary for object memory consolidation

This experiment was designed to determine the extent to which DHK-induced GLT-1 inhibition in the DH impaired OR and OP memory consolidation, and to identify a dose of DHK that did not impair memory on its own for use in the subsequent DHK-E_2 interaction study. Immediately after training in OR and OP, mice received a bilateral DH infusion of vehicle or one of three doses of DHK (1, 3.25, or 6 nM). During OR testing, mice treated with vehicle or 1 nM DHK spent significantly more time than chance with the novel object (vehicle: $t_{(8)} = 2.418$, $p = 0.042$, d = 0.81; 1 nM: $t_{(11)} = 2.304$, $p = 0.042$, d = 0.69; Fig. 1A), suggesting that 1 nM DHK did not impair within-group object recognition memory consolidation. In contrast, mice receiving 3.25 ($p = 0.237$) or 6 nM ($p = 0.764$) DHK spent chance amounts of time with the novel object (Fig. 1A), indicating that both doses impaired memory. A one-way ANOVA was not significant ($p = 0.425$).

During OP testing, mice treated with vehicle, 1 nM DHK, or 3 nM DHK spent significantly more time than chance with the moved object (vehicle: $t_{(8)} = 3.94$, $p = 0.004$, d = 1.31; 1 nM: $t_{(10)} = 2.348$, $p = 0.041$, d = 0.71; 3.25 nM: $t_{(8)} = 2.996$, $p = 0.017$, d = 1; Fig. 1B), indicating that both 1 and 3.25 nM DHK had no impairing effect on within-group spatial memory consolidation. However, mice receiving 6 nM DHK spent chance amounts of time with the moved object (Fig. 1B, $p = 0.11$), suggesting that this dose impaired spatial memory as well as object recognition. In addition, one-way ANOVA confirmed significant between-group differences in OP memory consolidation ($F_{(3,33)} = 5.331$, $p = 0.004$, $\eta_p^2 = 0.33$). Post hoc comparisons revealed that the 6 nM DHK group differed significantly from the vehicle ($p = 0.005$), 1 nM ($p = 0.014$), and 3.25 nM ($p = 0.026$) groups.

Collectively, these dose-response data suggest an important role of DH GLT-1 activity in the consolidation of spatial and object recognition memories in female mice and identified a behaviorally subeffective dose of DHK (1 nM) that could be used in combination with E_2 to determine the role of GLT-1 in the memory-enhancing effects of E_2.

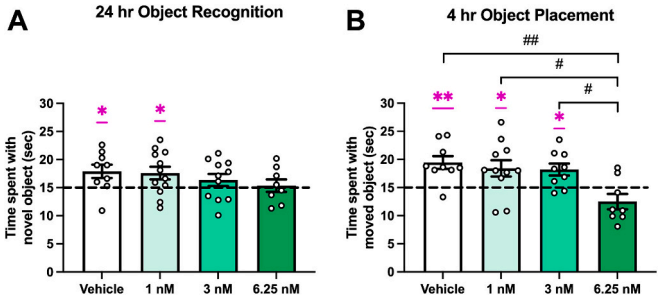

Fig. 1. The GLT-1 inhibitor DHK dose-dependently impaired memory consolidation in the object recognition (OR) and object placement (OP) tasks. (A) Twenty-four hours after OR training, mice treated with vehicle or 1 nM DHK spent significantly more time than chance (dashed line at 15 s) with the novel object (*$p < 0.05$), whereas mice treated with 3 or 6.25 nM DHK did not, suggesting that both the middle and high doses of DHK impaired object recognition memory consolidation. Five mice were excluded from drug infusion and testing because they did not reach the 30-s exploration criterion during OR training. (B) In OP, the only group that did not spend significantly more time than chance with the novel object 4 h after training was the 6.25 nM DHK group (*$p < 0.05$, **$p < 0.01$), indicating that only this high dose impaired spatial memory consolidation. This conclusion was supported by a significant main effect of treatment and post-hoc tests indicating that the 6.25 nM group spent less time with the novel object than all other groups (#$p < 0.05$, ##$p < 0.01$). Three mice were excluded from drug infusion and testing because they did not reach the 30-s exploration criterion during OP training. Bars represent the mean ± standard error of the mean (SEM).

3.2. The memory-enhancing effects of E_2 depend on GLT-1 activity

We next examined the extent to which GLT-1 activity is necessary for the memory-enhancing effects of E_2. As discussed above, we used the behaviorally subeffective 1 nM dose of DHK (Fig. 1) to ensure that potential memory impairments following DHK + E_2 infusion were due to an interaction between DHK and E_2 rather than a memory-impairing effect of DHK on its own. The results for OR and OP were nearly identical. As expected, mice receiving DH and ICV infusion of vehicle + vehicle or DHK + vehicle did not spend significantly more time than chance with either the novel object (Fig. 2A; vehicle + vehicle, $p = 0.654$, DHK + vehicle, $p = 0.834$) or moved object (Fig. 2B; vehicle + vehicle, $p = 0.634$, DHK + vehicle, $p = 0.667$) during testing. By contrast, mice receiving infusions of vehicle + E_2 spent significantly more time than chance with the novel object (Fig. 2A; $t_{(11)} = 3.244$, $p = 0.008$, d = 0.94) and moved object (Fig. 2B; $t_{(12)} = 3.326$, $p = 0.006$, d = 0.92) during testing. Strikingly, mice receiving infusions of DHK + E_2 did not spend significantly more time than chance with the novel object (Fig. 2A; $p = 0.117$) or moved object (Fig. 2B; $p = 0.462$) during testing.

A two-way ANOVA further confirmed a significant interaction between DHK and E_2 treatment for object recognition ($F_{(1, 41)} = 6.749$, $p = 0.01$, $\eta_p^2 = 0.14$), and post-hoc analysis showed that the vehicle + E_2 group spent significantly more time with the novel object than the DHK + E_2 group ($p = 0.014$). Combined, these data demonstrate that inhibition of GLT-1 activity in the DH blocks the memory-enhancing effects of E_2.

3.3. E2 rapidly activates ERK-associated signaling in hippocampal astrocytes, an effect dependent on GLT-1 activity

Lastly, because the memory-enhancing effects of E_2 are dependent on the rapid phosphorylation of the p42 isoform of ERK (p42 ERK) and Akt in the DH (Fernandez et al., 2008; Fortress et al., 2013), we next asked whether these phosphorylation events occur in DH astrocytes, and whether GLT-1 inhibition would prevent E_2 from increasing ERK and Akt activation in DH astrocytes. To assess the purity of magnetically sorted astrocyte fractions, pooled astrocytic lysate (lanes 1–6, $n = 3$–4/pool, Fig. 3A) was run concurrently with hippocampal lysate not subjected to cell sorting (lane 7, Fig. 3A). Astrocytic fractions (Fig. 3A, top) were positive for GFAP, an astrocytic protein, and negative for NeuN and Iba1, neuronal (Fig. 3A, middle) and microglial (Fig. 3A, bottom) proteins, respectively, indicating high purity of astrocytic samples.

For levels of Akt phosphorylation (Fig. 3B), the main effects of DHK ($F_{(1,8)} = 7.564$, $p = 0.03$, $\eta_p^2 = 0.49$) and E_2 ($F_{(1,8)} = 8.684$, $p = 0.02$, $\eta_p^2 = 0.52$) were significant, as was the DHK by E_2 interaction ($F_{(1,8)} = 11.52$, $p = 0.01$, $\eta_p^2 = 0.59$). Likewise, for p42 ERK phosphorylation (Fig. 3C), the main effects of DHK ($F_{(1,8)} = 24.02$, $p = 0.001$, $\eta_p^2 = 0.75$) and E_2 ($F_{(1,8)} = 30.73$, $p < 0.001$, $\eta_p^2 = 0.79$) were significant, as was the DHK by E_2 interaction ($F_{(1,8)} = 15.63$, $p = 0.004$, $\eta_p^2 = 0.66$). Tukey's post hoc analyses revealed that levels of phosphorylated protein in the vehicle + E_2 group were significantly higher than every other group for both p42 ERK ($p < 0.001$ vs vehicle + vehicle; $p < 0.001$ vs DHK + vehicle; $p = 0.001$ vs DHK + E_2) and Akt ($p = 0.01$ vs vehicle + vehicle; $p = 0.02$ vs DHK + vehicle; $p = 0.01$ vs DHK + E_2). p44 ERK phosphorylation (Fig. 3D) was not significantly affected by E_2 or DHK.

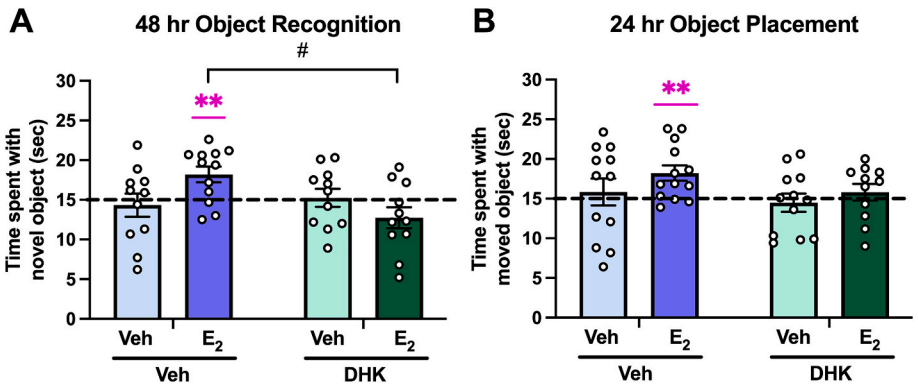

Fig. 2. GLT-1 inhibition blocked the beneficial effects of E_2 on memory consolidation. As expected for the longer 48 h and 24 h delays, mice receiving bilateral infusions of vehicle (veh) into the dorsal hippocampus (DH) and vehicle into the dorsal third ventricle (ICV) spent no more time than chance with the novel (A) or moved (B) objects, suggesting a lack of persistent memory for the training objects beyond the 24 and 4 h time points tested in Fig. 1. In contrast, mice receiving DH vehicle + ICV E_2 spent significantly more time than chance (**$p < 0.01$) with the novel and moved objects, suggesting the E_2 alone enhanced memory in both tasks relative to chance. However, DHK blocked these memory-enhancing effects, as mice receiving DH infusion of the behaviorally subeffective 1 nM DHK plus ICV vehicle did not display memory for the training objects in either task. In OR, vehicle + E_2 group also spent significantly more time with the novel object than the DHK + E_2 group (#$p < 0.05$), as assessed by a posthoc test based on the significant interaction between DHK and E_2 treatments. Twelve mice in OR and 9 mice in OP were excluded from drug infusion and testing because they did not reach the 30-s exploration criterion during training. Bars represent the mean ± SEM.

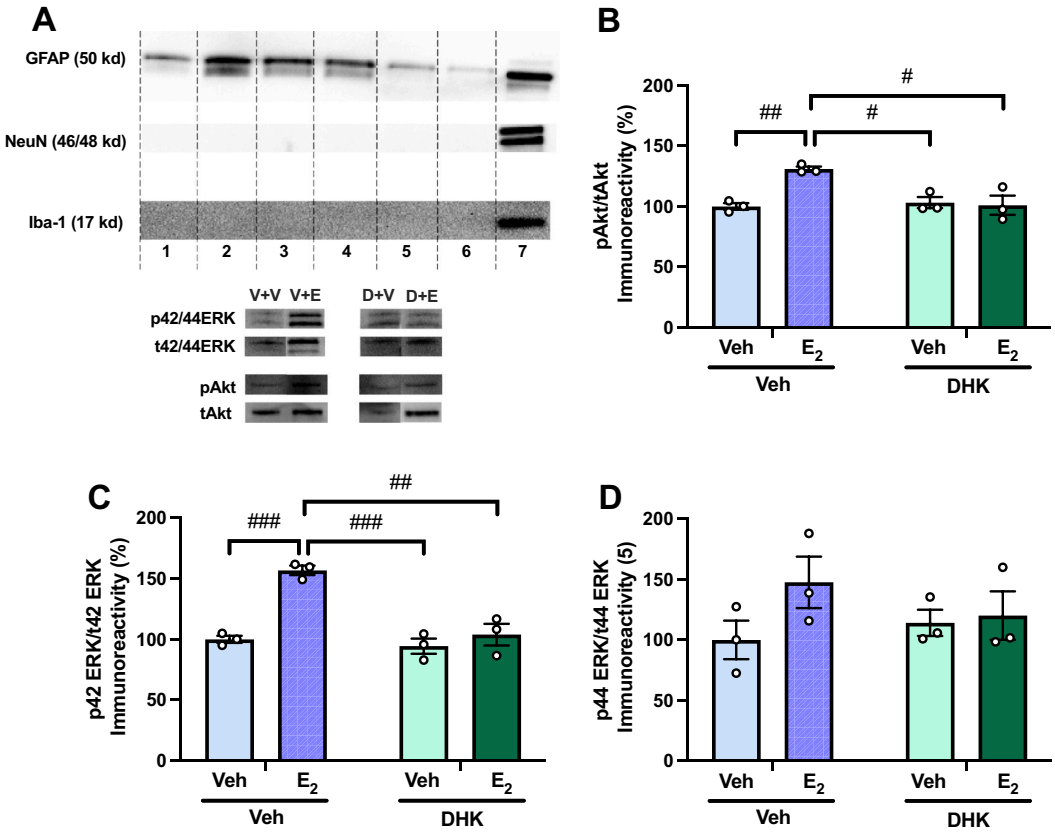

Fig. 3. The E_2-induced increase in astrocytic p42 ERK and Akt was blocked by DHK. (A) Western blotting of GFAP, NeuN, and Iba1 in magnetically sorted astrocyte fractions from pooled astrocytic lysate (lanes 1–6) and hippocampal lysate not subjected to cell sorting (lane 7). GFAP staining was found exclusively in astrocyte fractions. (B, C) E_2 significantly increased levels of phosphorylated Akt and p42 ERK in mice receiving DH infusion of vehicle (#$p < 0.05$, ##$p < 0.01$; ###$p < 0.01$), but not DHK, indicating that DHK blocked the effects of E_2 on protein phosphorylation. (D) Neither E_2 nor DHK significantly affected p44 ERK phosphorylation. Bars represent the mean ± SEM.

These results indicate that levels of Akt (Fig. 3B) and p42 ERK (Fig. 3C) phosphorylation were increased in magnetically cell-sorted DH astrocytes 5 min following DH E_2 infusion. E_2-induced phosphorylation events were blocked in DH astrocytes by DHK. Together, these data suggest that E_2 can robustly and rapidly increase p42 ERK and Akt activation in DH astrocytes, and that these effects depend on DH GLT-1.

4. Discussion

Although estrogens often facilitate memory consolidation (Frick,

2015; Luine et al., 2018; Sheppard, 2018; Taxier et al., 2020), the roles of specific cell types in mediating this effect are largely unknown. Perhaps due to early evidence that E_2 increases CA1 pyramidal neuron dendritic spine density and potentiates synaptic plasticity (Gu and Moss, 1996; Woolley et al., 1997; Woolley and McEwen, 1993; Woolley and McEwen, 1992), studies of estrogen's effects on memory have focused almost entirely on excitatory neurons, despite abundant in vitro data that E_2 promotes astrocyte activity (Acaz-Fonseca et al., 2014; Azcoitia et al., 2010; Crespo-Castrillo et al., 2020). Recent reports demonstrate that astrocytes play a central role in memory formation (Adamsky et al., 2018; Kol et al., 2020), begging the question of their role in mediating the memory-enhancing effects of E_2. Results from this study suggest that estrogenic regulation of memory consolidation and cell signaling in the DH depends upon GLT-1. Although the possibility exists that GLT-1 expression in neurons could also play a role in regulating memory formation both in the presence and absence of estrogens, the predominance of GLT-1 expression in astrocytes suggests a prominent astrocytic role. Additionally, the present study implicates astrocytes as a novel site of memory-related estrogenic action by demonstrating that antagonism of DH GLT-1 prevents estrogens from enhancing memory consolidation via blockade of rapid p42 ERK and Akt phosphorylation in astrocytes.

The finding that GLT-1 blockade in the dorsal hippocampus impaired object memory consolidation in females is consistent with existing studies of male rodents demonstrating that ICV GLT-1 infusion impairs object recognition (Tian et al., 2019) and spatial memory tested in the Morris water maze (Bechtholt-Gompf et al., 2010). Our data extend this work by showing that direct intrahippocampal infusion of DHK, rather than central administration, impairs both object recognition and object placement memory consolidation. Because blockade of GLT-1 impairs astrocytic ability to clear glutamate from the synaptic cleft, the observed impairments in object recognition and object placement memory in the present study perhaps resulted from an excess of extracellular glutamate, thereby potentiating inappropriate tuning of excitatory and inhibitory balance within the DH. This explanation is parsimonious with the necessity of hippocampal glutamate transporters for maintaining input specificity (Arnth-Jensen et al., 2002; Diamond, 2001) and with previous in vivo work showing that DHK-induced increases in hippocampal glutamate levels were associated with increased neuronal excitability (Muñoz et al., 1987).

The present data are also the first to show an essential role for GLT-1 in memory among females. Although our primary question focused on the role of GLT-1 in mediating E_2's effects on memory, comparing GLT-1 data collected in both sexes is important because previous work has shown that females have greater GFAP immunoreactivity and greater astrocyte size than males in hippocampal subregions CA1, CA3, and dentate gyrus (Arias et al., 2009). A more recent in-depth quantitative proteomics analysis of neonatal rat hippocampal organotypic slice cultures also demonstrated greater astrocytic metabolism and expression of astrocytic markers, including GFAP, in females relative to males (Weis et al., 2021). These sex differences in hippocampal astrocytes could potentially lead to disparate astrocytic involvement in memory consolidation between the sexes. Although our data and previous DHK studies in males suggest similar involvement of GLT-1 function in mediating hippocampus-dependent memory among males and females, future work should more directly compare both sexes in the same experimental conditions to assess potential sex differences in astrocytic memory modulation.

One key finding of the present work is the necessity of GLT-1 for estrogenic enhancement of memory consolidation. Although E_2 is an established regulator of GLT-1 expression (Karki et al., 2014; Lee et al., 2012b; Lu et al., 2020; Pawlak et al., 2005), our data are the first to suggest that the memory-enhancing effects of E_2 are dependent upon activity of DH GLT-1. Given that E_2 has been shown to increase levels of GLT-1 protein and mRNA (Lee et al., 2012b; Pawlak et al., 2005), we posit that one mechanism underlying estrogenic memory enhancement in the present study is an E_2-facilitated increase in GLT-1 expression, which augments the ability of DH astrocytes to clear extracellular glutamate, thereby promoting adaptive hippocampal synaptic plasticity. A potential mechanism underlying this increased plasticity is increased astrocytic cell signaling, as indicated by our finding that DH E_2 infusion increased phosphorylation of p42 ERK and Akt within hippocampal astrocytes. These data are consistent with work demonstrating increased Akt phosphorylation (Bains and Roberts, 2016) and p42 ERK phosphorylation (Lee et al., 2012b) in astroglial cultures following treatment with E_2. In midbrain astrocyte cultures, actions of ERα at the plasma membrane appear to mediate the E_2-induced increased in ERK phosphorylation (Pawlak et al., 2005). Importantly for the present study, ERK and Akt phosphorylation are necessary for E_2 to increase GLT-1 expression in primary cortical astrocyte cultures (Lee et al., 2012a), suggesting reciprocal interactions between astrocytic cell signaling and GLT-1 expression that could contribute to DHK's ability to block E_2-induced p42 ERK and Akt activation in our DH astrocytes. However, it is important to note that our findings in isolated astrocytes cannot exclude possible effects in other cell types, including neurons, because our cell-sorting method did not permit examination of p42 ERK and Akt phosphorylation in the remaining astrocyte-negative fraction. To more definitively exclude effects on neurons and other cell types, these must be isolated and tested in future studies.

How might E_2 interact with GLT-1 to mediate memory? Astrocytes express a multitude of G-protein-coupled receptors (GPCRs), including mGluR1a, which is notable given its necessity for the memory-enhancing effects of E_2 (Boulware et al., 2013). Given that the physical association of mGluR1a and ERα in hypothalamic astrocytes initiates intra-astrocytic calcium release (Kuo et al., 2010), similar processes could be at play in the hippocampus. This hypothesis is supported by additional work linking the activation of ERα to mGluR1a signaling and downstream ERK phosphorylation in vitro and in vivo in the hippocampus (Boulware et al., 2013; Boulware et al., 2005), although it remains unclear the extent to which this happens in neurons, astrocytes, or both. Thus, interplay between astrocytic mGluR1 and astrocytic estrogen receptors following E_2 treatment may facilitate intracellular signaling within hippocampal astrocytes to promote memory consolidation. Because our work suggests that activity of GLT-1 is critical for the memory-enhancing effects of E_2, an additional mechanism at play may be competition between GLT-1 and mGluR1a for extracellular glutamate (Huang et al., 2004). If intrahippocampal E_2 increases hippocampal expression of GLT-1, as it does in cultured midbrain astrocytes (Pawlak et al., 2005), perhaps the balance of synaptic glutamate clearance shifts further toward being GLT-1-dependent, rather than mGluR-dependent, thus freeing more mGluRs to associate with membrane ERα and initiate intracellular signaling. Other astrocytic GPCRs may be involved in the facilitatory effects of E_2 on memory consolidation, although this remains to be rigorously tested. Broadly, however, these data support the hypothesis that E_2 initiates similar GPCR-coupled signaling cascades in astrocytes, as it is presumed to do in neurons, to facilitate memory consolidation.

Taken together, the results from the present study suggest that hippocampal astrocytes are essential for memory consolidation in females and for E_2's facilitatory effects on memory consolidation. Specifically, astrocytic glutamate reuptake in the DH is critical for the memory-enhancing effects of E_2, and intrahippocampal E_2 facilitates rapid p42 ERK and Akt phosphorylation in DH astrocytes in a GLT-1-dependent manner. Because astrocytes are newly emergent players in estrogenic enhancement of memory formation, our results invite a multitude of additional questions. Future work should consider sex differences in estrogen-astrocyte interactions, given that the present study only examined female mice. Disentangling the pro-cognitive effects of E_2 on hippocampal astrocytes versus neurons is another important next step, as is determining astrocyte-neuron interactions following E_2 administration. Finally, understanding the roles of specific astrocyte-expressed GPCRs in E_2-facilitated memory consolidation could provide novel insights into potential druggable targets that may help alleviate cognitive

dysfunction. Ultimately, our findings suggest that the mnemonic benefits of E_2 extend beyond their capacity to influence neurons, and highlight the need to appreciate astrocytes, once relegated to the sidelines, as key regulators of estrogenic memory processes.

Author contributions

LRT, KMF, and FS designed the project and experiments. TEB and FS supervised and trained LRT on magnetic-activated cell sorting of astrocytes. LRT and MP performed the behavioral experiments, magnetic-activated cell sorting, and Western blotting. LRT performed data analysis for the above experiments. LRT and KMF wrote the first draft of the manuscript, with editing and reviewing by MP, TEB, and FS.

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CRediT authorship contribution statement

Lisa R. Taxier: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Miriam Pillerová:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Taylor E. Branyan:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Farida Sohrabji:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Karyn M. Frick:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

Dr. Frick is a co-founder and the Chief Scientific Officer of 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. The remaining authors have no conflicts of interests to declare.

Data availability

Data will be made available on request.

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