Neurobiology of Aging 78 (2019) 111–120

Contents lists available at ScienceDirect

Neurobiology of Aging

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

CNS luteinizing hormone receptor activation rescues ovariectomy-related loss of spatial memory and neuronal plasticity

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ARTICLE INFO

Article history:
Received 10 July 2018
Received in revised form 31 January 2019
Accepted 1 February 2019
Available online 13 February 2019

Keywords:
Luteinizing hormone
Luteinizing hormone receptor
Cognition
Neuronal plasticity
Ovariectomy
Menopause

ABSTRACT

Ovariectomy (OVX), a menopause model, leads to cognition and neuronal plasticity deficits that are rescued by estrogen administration or downregulation of pituitary luteinizing hormone (LH). LH is present in the brain. However, whether LH levels differ across brain regions, change across reproductive stages, or whether brain-specific LHR signaling play a role in OVX-related cognitive and neuroplasticity losses is completely unknown. To address this, we measured brain LH in cycling and OVX C57Bl/6 across brain regions and determined whether OVX-related functional and plasticity deficits could be rescued by intracerebroventricular administration of the LHR agonist (hCG). Here, we show that while pituitary LH is increased in OVX, brain LH is decreased, primarily in spatial memory and navigation areas. Furthermore, intracerebroventricular hCG delivery after OVX rescued dendritic spine density and spatial memory. In vitro, we show that hCG increased neurite outgrowth in primary hippocampal neurons in a receptor-specific manner. Taken together, our data suggest that loss of brain LH signaling is involved in cognitive and plasticity losses associated with OVX and loss of ovarian hormones.

1. Introduction

Age-related hypothalamic-pituitary-gonadal (HPG) axis hormone level changes are linked to cognitive decline in humans (Henderson and Sherwin, 2007; Rocca et al., 2007) as well as in rodents (Blair et al., 2016; Bryan et al., 2010; Heikkinen et al., 2004; Palm et al., 2014; Telegdy et al., 2009; Ziegler and Thornton, 2010). Furthermore, the different patterns of loss of sex steroids between men and women, with the latter being more abrupt, may be an important factor in the gender disparity in Alzheimer's disease (AD) risk (Zandi et al., 2002). Epidemiological studies have highlighted the protective nature of hormone replacement therapy (HRT) against menopausal cognitive decline and AD (Kawas et al., 1997; Tang et al., 1996; Zandi et al., 2002). However, the benefits of HRT seem to be restricted to a critical period in both women and rodents (Benson et al., 2010; Blair et al., 2016; Chlebowski et al., 2010; Daniel and Bohacek, 2010; Daniel et al., 2006; Luine and Rodriguez, 1994; Rapp et al., 2003).

Importantly, during menopause, there is not only a loss of sex steroid production but also an increase in the peripheral (serum) levels of the gonadotropin luteinizing hormone (LH), which has also been shown to be relevant to cognition and plasticity. To this end, increases in serum LH correlate with AD risk (Butchart et al., 2013; Hogervorst et al., 2004; Short et al., 2001; Verdile et al., 2014) as well as cognitive deficits in the elderly (Hyde et al., 2010) and rodents (Barron et al., 2010; Berry et al., 2008; Casadesus et al., 2007). Furthermore, several studies now show pharmacological downregulation of peripheral LH in rodents is as effective as estrogen replacement in improving cognitive loss (Blair et al., 2016; Bowen et al., 2015; Bryan et al., 2010; Casadesus et al., 2007; Palm et al., 2014; Ziegler and Thornton, 2010). Importantly, the benefits of lowering peripheral LH are maintained beyond the critical period of estrogen treatment (Blair et al., 2016), thus highlighting the central role of LH in regulating cognition.

Central to the ability of LH to affect spatial memory, the receptor (LHR), also activated by hCG, is expressed in the brain and present in cognitive-related areas within the cortex as well as in the hippocampus (Lei et al., 1993; Apaja et al., 2004). The functionality of the LHR in the brain has been shown in vitro (Al-Hader et al., 1997a, b) and in vivo, by increasing multiunit recording activity in the hippocampus (Gallo et al., 1972), where LHR activation has been linked to courtship behavior (Yang et al., 2007), pheromone-related increases in hippocampal neurogenesis (Mak et al., 2007), and regulation of activity and anxiety (Lukacs et al., 1995). However, whether LHR activation is associated with functional and plasticity loss or rescue is yet to be clearly determined.

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0197-4580/$ – see front matter © 2019 Elsevier Inc. All rights reserved.
https://doi.org/10.1016/j.neurobiolaging.2019.02.002

Studies that show downregulation of peripheral LH leads to cognitive improvements (Blair et al., 2016; Bryan et al., 2010; Palm et al., 2014; Ziegler and Thornton, 2010) and administration of LHR agonists leads to cognitive loss (Berry et al., 2008; Burnham et al., 2017) support the hypothesis that activation of LHR may be detrimental. On the other hand, the mechanism through which this may occur is unclear. First, LH is a large glycoprotein that is unlikely to penetrate the blood-brain barrier (BBB) without a transporter molecule, and evidence to this end is thus far contradictory, showing both the ability of LH to cross the BBB (Banks et al., 1993) as well as the lack of ability to cross the BBB (Ondo et al., 1972). In addition, LH is present in brain (Emanuele et al., 1983; Glass and McClusky, 1987), and these levels may show an inverse relationship to LH in the periphery (Emanuele et al., 1981; Palm et al., 2014). Thus, this suggests high levels of LH in the periphery may either restrict transport of LH into the brain or reduce transcription and/or translation of LH endogenously produced in the brain, ultimately leading to dysfunction.

Furthermore, signaling mechanisms of LHR activation support a beneficial, not a detrimental role of LHR on cognition and plasticity. To this end, the LHR is a G-protein–coupled receptor that signals through Gs, thus activating extracellular signal-related protein kinase (ERK) or the activating protein kinase A (Meng et al., 2007; Menon and Menon, 2012); both are critical cascades in long-term potentiation, memory, and structural plasticity (Bach et al., 1999; Blum et al., 1999; Briz et al., 2013; English and Sweatt, 1997; Goldin and Segal, 2003; Hardingham et al., 2001; Hebert and Dash, 2002; Impey et al., 1998; Selcher et al., 1999; Wu et al., 2001; Zadran et al., 2009). LHR activation is also known to signal through Gq, driving GSK3β inhibition and β-catenin activation (Breen et al., 2013; Palm et al., 2014), which is also known to be beneficial for cognition and plasticity and is involved in AD neuroprotection (Graham et al., 2015; He and Shen, 2009; Kleppisch et al., 2001; Kwok et al., 2008; McQuail et al., 2013). Lastly, the LHR is internalized in the presence of high levels of its ligand (Hu et al., 1990; Kishi et al., 2001; Min et al., 1998; Peegel et al., 1994; Segaloff et al., 1990), suggesting dysfunction associated with LHR activation (Berry et al., 2008; Burnham et al., 2017; Ziegler and Thornton, 2010) could be mediated through downregulation of LHR levels rather than activation. Therefore, given the considerations above, a closer study of brain LH, its relationship to peripheral LH, and its ability to regulate cognition and plasticity are warranted. The underlying hypothesis for this study was that loss of brain LH, driven by high peripheral LH in menopausal states, leads to loss of LHR signaling and cognitive loss that can be rescued by directly administering low, nonreceptor downregulating doses of an LHR agonist.

2. Methods

2.1. Animals

Female C57Bl/6J mice (Jackson Laboratories; Bar Harbor, ME; Stock 000664) were housed in accordance with the Kent State University Institutional Animal Care and Use Committee. Housing included water and food *ad libitum* as well as a 12 hours light:dark cycle starting at 08:00. Animals were ovariectomized at 3 months of age, and at 6 months of age, animals were either sacrificed for brain LH measurements or implanted with a brain infusion cannula (Alzet; Brain Infusion Kit 3) in the right lateral ventricle that connected to an osmotic pump (Alzet; #1004) delivering hCG or vehicle. A 3-month delay between ovariectomy (OVX) and treatment onset was used to ensure that brain LH levels were reduced as previously described (Palm et al., 2014).

2.2. Bilateral ovariectomy

Mice underwent bilateral ovariectomy at 3 months of age as previously described (Blair et al., 2016). Briefly, once the proper plane of anesthesia was reached using isoflurane, a small incision was made and both ovaries were removed. Control animals (SHAM) animals underwent identical procedures, but the ovaries were exposed and placed back in the abdominal cavity rather than being resected. Wound clips closed the incision, and the animals were placed in clean cages to recover and subsequently moved back to their housing rooms.

2.3. Cannula implantation

Stereotaxic surgery was performed when animals were 6 months of age. Osmotic pumps were primed with artificial cerebrospinal fluid (aCSF), 3 mIU hCG/day or 30mIU hCG/day (Sigma; C8554-50IU) per the manufacturer's instructions. The cannula was stereotaxically placed to deliver in the right lateral ventricle (AP = −0.5 ML = −1.1 DV = −2.5) where it was cemented to the skull and a screw. The osmotic pump and tubing connected to the cannula were placed subcutaneously. All animals had properly placed cannula as verified by injecting fast green through the tubing or observing the cannula track through the tissue via cryosectioning.

2.4. Serum analysis

Blood was drawn from a terminal cardiac puncture, stored overnight at 4 °C, and then spun at 5000 rpm for 30 minutes at 4 °C. Serum was collected and stored at −20 °C until all samples were collected. Serum was diluted (12 μL serum in 108 μL assay buffer: 0.2% BSA − 0.05% Tween 20 − PBS) for LH measurements, and undiluted serum was used for 17β-estradiol (E2) and progesterone (p4) measurements. Samples were analyzed at The University of Virginia (UVA) Center for Research in Reproduction Ligand Assay and Analysis Core using the ultra-sensitive mouse and rat LH ELISA, mouse and rat—estradiol ELISA (Calbiotech), and mouse and rat-progesterone ELISA (IBL) assay.

2.5. Tissue analysis

Three months after OVX the animals were decapitated, and several regions of the brain were collected and stored at −80 °C. Brain regions were homogenized in assay buffer and centrifuged. The supernatant was stored at −20 °C and the pellet was resuspended in assay buffer, incubated overnight at 4 °C, and centrifuged. The second supernatant was combined with the first. Protein was sent to UVA for analysis of the pituitary, hypothalamus, cerebellum, cingulate cortex, temporal cortex, hippocampus, prefrontal cortex, and basal ganglia/thalamus.

2.6. Morris water maze

The Morris water maze task was used to measure spatial learning and memory. A pool of opaque white water was temperature controlled, and a platform was placed hidden below the surface of the water in the target quadrant. Animals were trained to swim then placed facing the wall in the pool and tracked for 60 seconds or 2 seconds time passed with them on the hidden platform. There were 4 trials per day for 5 days of training, and the animal was placed in a different quadrant for each trial of the day to discourage path-based learning. Three training trials were performed on the 6th day followed by a probe trial where the hidden platform was removed, and the animal was forced to swim for

60 seconds. Testing for visual capabilities was performed with a visible platform on day 7. All animals passed the visual test; however, 3 SHAM+aCSF animals were removed because of behavior discordant with spatial memory testing. Two were removed for circling the perimeter of the pool for multiple trials per day on multiple days, and 1 was removed for failing to train to stand on the platform.

2.7. Dendritic spine density

The hemibrains from perfused (1.5% paraformaldehyde) animals were postfixed 4 hours in 1.5% paraformaldehyde and then stored at −20 °C in 30% sucrose: 30% glycerol: 0.2% sodium azide. The hemisphere was then thawed over 24 hours and switched to phosphate buffer for an additional 48 hours all at 4 °C. Hemispheres were stained using the FD Rapid GolgiStain kit (FD NeuroTechnologies) using kit instructions. Sections were cut 150 μm on a cryostat before being mounted on gelatin-coated slides (FD NeuroTechnologies). Dendritic segments from layer II/III pyramidal cells of the retrosplenial cortex were taken with a 60X oil objective (Olympus IX81) to create Z-stack images at 0.27 μm. Analysis occurred on apical dendrites traced from the soma for the first 45 μm, and spines were detected using NeuronStudio (Mount Sinai School of Medicine) from 4 to 6 cells per animal.

2.8. Western blotting

Cell lysis buffer (Cell Signaling Technology) was used to homogenized hippocampi and cortices. Protein samples were separated using SDS-PAGE with Tris-tricine gels. Protein was transferred to PVDF membranes (Millipore) and blocked in 10% milk for 1 hour before being incubated with primary antibodies in TBS-T: LHR (1:1000; Santa Cruz Biotechnology), pERK (1:1000; Cell Signaling Technology), ERK (1:1000; Santa Cruz Biotechnology), synaptophysin (1:1000; Cell Signaling Technology), glyceraldehyde 3-phosphate dehydrogenase ([GAPDH]; 1:25,000; Sigma), and actin (1:50,000; Millipore). Membranes were washed and secondary antibody with horseradish peroxidase (1:1000; Cell Signaling Technology) was applied for 1 hour. The PXi imaging system (Syngene) was used to capture images, and ImageJ software (NIH) was used to calculate optical density.

2.9. Neurite outgrowth

Hippocampi from E18 mouse embryos were purchased from BrainBits LLC and grown on 12 mm poly-d-lysine–coated glass coverslips in Neurobasal media (B27, glutamax, Pen/Strep, Fungizone) in a 5% CO_2, 37 °C humidified chamber. A total of 6 coverslips per group from multiple experiments were treated independently with either control media, 300 mIU/mL hCG (Sigma: C8554-50IU) or 300mIU/mL deglycosylated hCG on DIV1 and DIV3. Neurons were fixed with 4% paraformaldehyde on DIV4 and then blocked in 10%NGS-0.3%triton-X100 in PBS and incubated in anti-MAP2 primary antibody (Cell Signaling Technology; 1:200%, 1% NGS in PBS) overnight. Coverslips were washed and incubated in Alexafluor 594 secondary antibody (Molecular Probes; 1:400; 1%NGS in PBS) and then mounted to slides and imaged via fluorescent microscopy (Leica DM4000 B). NeuronStudio (Mount Sinai School of Medicine) was used to trace individual neurons and perform analysis of morphology. The number of neurites from the soma (primary neurites), the number of higher order neurites (nerve terminals minus primary neurites), and the number of branch points were analyzed.

2.10. Statistical analysis

Analysis was performed with SPSS 24 (IBM). Hormone levels for SHAM and OVX were compared using Student's t test with Cohen's d for effect size. Repeated measures ANOVA with partial η^2 for effect size was used to analyze for Morris water maze training. Pearson correlation was performed for the relationship between spine density and Morris water maze probe data. All other analyses were performed as 1-way ANOVAs with Fisher's LSD post hoc comparisons with η^2 as the measure of effect size.

3. Results

3.1. Luteinizing hormone levels in the brain and periphery after ovariectomy

We analyzed levels of LH in SHAM and OVX mice in the cerebellum, cingulate cortex, hippocampus, temporal cortex, prefrontal cortex, basal ganglia/thalamus, hypothalamus, and pituitary (Fig. 1A) to address whether LH in the brain changes due to reproductive senescence. LH levels were significantly lower by Student's t test in OVX as compared to SHAM in cingulate cortex ($t(33) = 8.208$, $p = 1.83 \times 10^{-9}$, $d = 2.12$), hippocampus ($t(36) = 9.083$, $p = 7.93 \times 10^{-11}$, $d = 2.46$), temporal cortex ($t(37) = 8.319$, $p = 5.37 \times 10^{-10}$, $d = 1.04$), and prefrontal cortex ($t(36) = 2.266$, $p = 0.030$, $d = 1.30$). As expected, pituitary LH levels were significantly increased in OVX as compared to SHAM ($t(38) = -6.077$, $p = 4.56 \times 10^{-7}$, $d = 1.65$). Lastly, there was no significant difference in LH levels between SHAM and OVX in the cerebellum ($t(30) = 1.036$, $p = 0.308$, $d = 0.47$), basal ganglia/thalamus ($t(8) = 1.059$, $p = 0.321$, $d = 0.55$), or hypothalamus ($t(39) = 1.095$, $p = 0.280$, $d = 0.36$). As expected with OVX, serum levels of LH (Fig. 1B) analyzed by Student's t test were significantly increased in OVX as compared to SHAM ($t(37) = -6.558$, $p = 1.11 \times 10^{-7}$, $d = 2.71$).

We analyzed the ratio of brain LH to pituitary LH (Fig. 1C) to investigate the relationship between pituitary LH and brain LH levels. To this end, the ratio of brain LH to pituitary LH was significantly lower in OVX as compared to SHAM in cingulate cortex ($t(38) = 2.064$, $p = 0.046$, $d = 0.98$), hippocampus ($t(30) = 5.363$, $p = 8.76 \times 10^{-6}$, $d = 1.39$), temporal cortex ($t(29) = 5.801$, $p = 2.74 \times 10^{-6}$, $d = 1.53$), prefrontal cortex ($t(29) = 4.928$, $p = 3.12 \times 10^{-5}$, $d = 1.30$), and hypothalamus ($t(37) = 4.474$, $p = 6.92 \times 10^{-5}$, $d = 1.21$). There was, however, no significant difference in the ratio of brain LH to pituitary LH in the cerebellum ($t(29) = 1.769$, $p = 0.087$, $d = 1.02$) or basal ganglia/thalamus ($t(6) = 2.297$, $p = 0.060$, $d = 1.22$). To verify proper OVX, we measured serum E2 (Fig. 1D). As expected, Student's t showed serum E2 was significantly lower in OVX compared to SHAM ($t(37) = 4.660$, $p = 4.02 \times 10^{-5}$, $d = 1.90$).

3.2. Spatial memory performance with intracerebroventricular (ICV) hCG after ovariectomy

To determine whether LHR activation in the CNS can rescue spatial memory deficits, we analyzed spatial memory effects of 2 doses of hCG treatment administered ICV to OVX mice (Fig. 2). Repeated measures ANOVA showed no significant differences in escape latency during training ($F(15, 250) = 1.107$, $p = 0.350$, partial $\eta^2 = 0.062$). One-way ANOVA for the probe trial shows a significant difference in the percent time spent in the target quadrant ($F(3, 50) = 3.970$, $p = 0.013$, $\eta^2 = 0.192$). Post hoc analysis demonstrates that SHAM+aCSF ($p = 0.002$, $d = 1.04$) and OVX+3mIU hCG ($p = 0.025$, $d = 0.60$) spent a higher percentage of time in the target quadrant compared to OVX+aCSF, whereas OVX+30mIU hCG was not significantly different from OVX+aCSF ($p = 0.076$, $d = 1.02$).

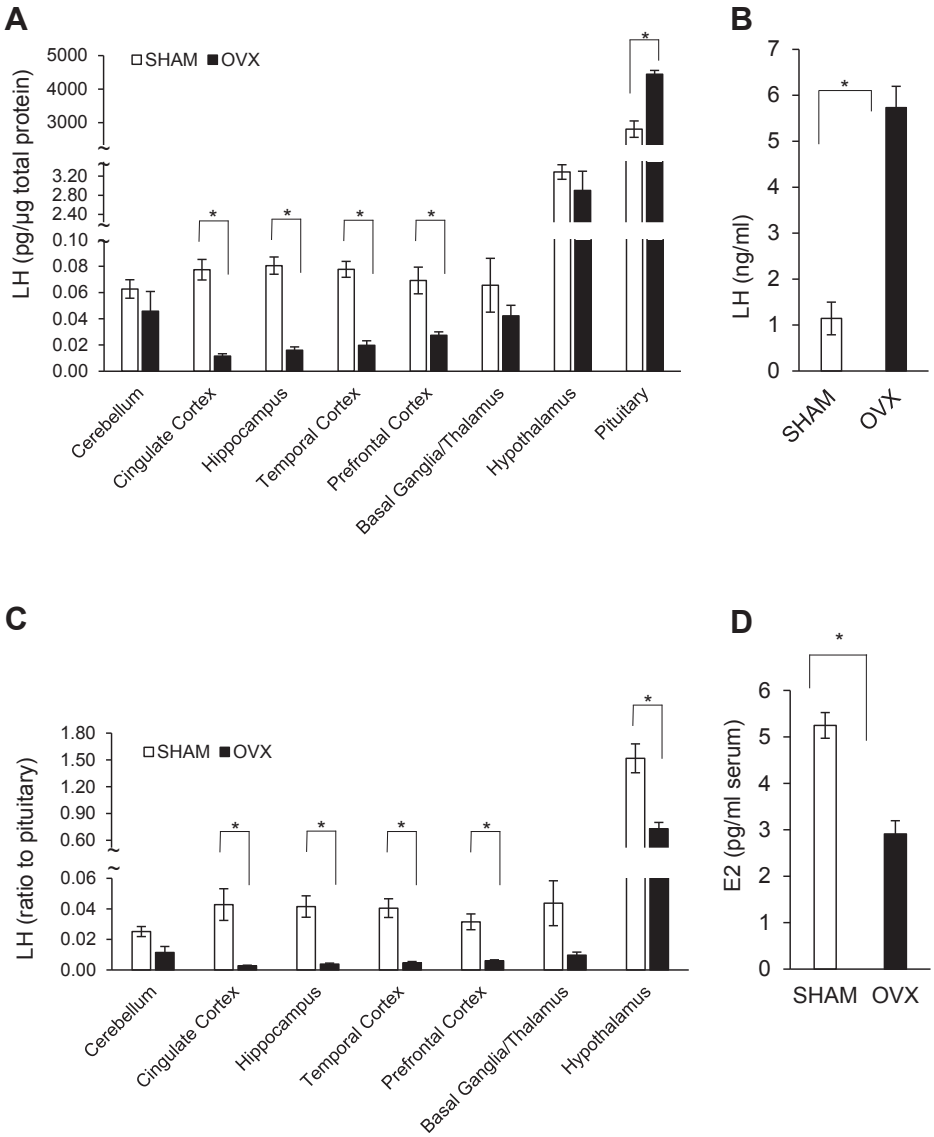

Fig. 1. LH levels in the brain of healthy cycling and ovariectomized mice. There were significantly lower LH levels in OVX as compared to SHAM in the cingulate cortex, hippocampus, temporal cortex, and prefrontal cortex. Levels of LH in pituitary were significantly higher in OVX as compared to SHAM (A). There were significantly higher levels of LH in OVX as compared to SHAM in serum (B). Cingulate cortex, hippocampus, temporal cortex, prefrontal cortex, and hypothalamus had significantly lower ratios of LH to pituitary LH in OVX as compared to SHAM (C). There was a significant decrease in E2 in OVX as compared to SHAM (D). Graphs represent average ±standard error of the mean. (* denotes $p < 0.05$ for Student's t, cerebellum SHAM n = 26, OVX n = 6; cingulate SHAM n = 31, OVX n = 10; hippocampus SHAM n = 30, OVX n = 10; temporal cortex SHAM n = 29, OVX = 10; prefrontal cortex SHAM n = 29, OVX = 9; basal ganglia/thalamus SHAM n = 7, OVX = 10; hypothalamus SHAM n = 31, OVX n = 10; pituitary SHAM n = 31, OVX = 9; serum LH SHAM n = 30, OVX n = 9; serum E2 SHAM n = 29, OVX n = 10). Abbreviations: LH, luteinizing hormone; OVX, ovariectomy.

Serum LH was analyzed to verify proper OVX (Table 1). There were higher levels of serum LH in OVX+aCSF ($p = 0.00011$, d = 3.12), OVX+3mIU hCG ($p = 0.00011$, d = 2.05), and OVX+30mIU hCG ($p = 1.2 \times 10^{-6}$, d = 2.30) as compared to SHAM+aCSF as shown by 1-way ANOVA (F (3,52) = 12.098, $p = 4.0 \times 10^{-6}$, $\eta^2 = 0.411$).

3.3. Dendritic spine density with ICV hCG after ovariectomy

The brain from OVX mice-treated ICV with hCG was Golgi stained, and dendritic spine density was measured in retrosplenial cortex to determine whether LHR activation in the CNS alters structural plasticity (Fig. 3 & Table 2). There was a significant difference by 1-way ANOVA in the number of total spines (F (3,18) = 3.609; $p = 0.038$, $\eta^2 = 0.419$) and stubby spines (F (3.18) = 4.209; $p = 0.024$; $\eta^2 = 0.457$). Post hoc analysis revealed a higher number of total spines in SHAM+aCSF ($p = 0.012$, d = 1.885) and OVX+30mIU hCG ($p = 0.017$, d = 1.892) as compared to OVX+aCSF as well as higher number of stubby spines SHAM+aCSF ($p = 0.008$, d = 1.865) and OVX+30mIU hCG ($p = 0.012$, d = 2.154) as compared to OVX+aCSF. We used a Pearson correlation to determine whether spatial memory correlated with dendritic spine density (Fig. 3B) and found a significant positive correlation for stubby dendritic spine density and percent time in the target quadrant of Morris water maze (r = 0.673, $p = 0.002$). There were no differences by 1-way ANOVA in the number of thin spines (F (3,16) = 0.047; $p = 0.986$, $\eta^2 = 0.009$) and mushroom spines (F (3,16) = 0.158; $p = 0.923$, $\eta^2 = 0.029$).

3.4. Cellular signaling with ICV hCG after ovariectomy

LHR activation can lead to desensitization via internalization and degradation of the receptor (Hu et al., 1990; Kishi et al., 2001; Min et al., 1998; Peegel et al., 1994; Segaloff et al., 1990).

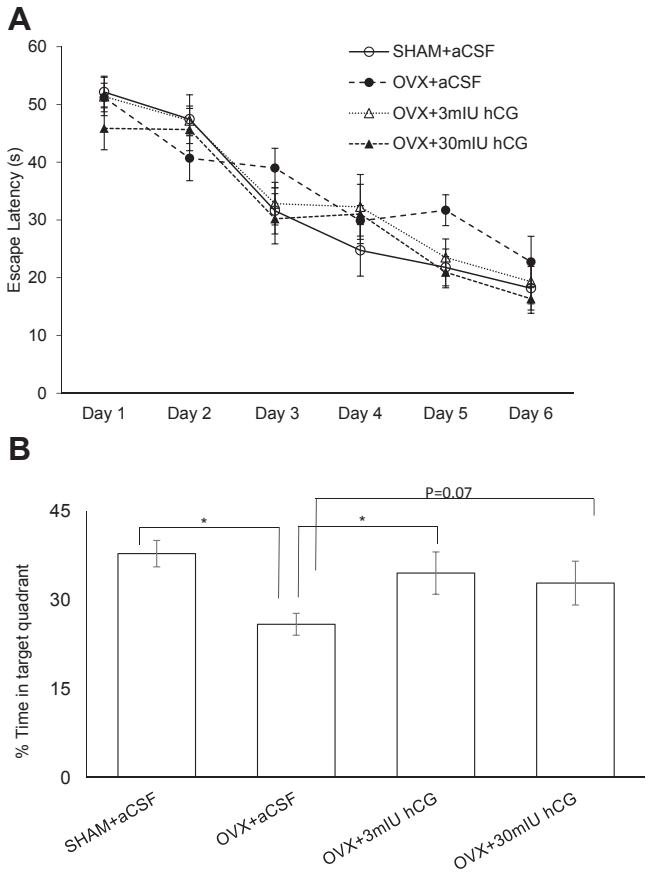

Fig. 2. Spatial memory of healthy cycling and ovariectomized mice treated ICV with aCSF or hCG. Spatial memory was analyzed as (A) escape latency and (B) percent time in the target quadrant of the Morris water maze task. There were no statistically significant differences during training sessions in escape latency across groups. In the probe trial, percent time in the target quadrant was significantly different by 1-way ANOVA. SHAM+aCSF and OVX+3mIU hCG spent more time in the target quadrant than OVX+aCSF. There was a trend toward significance for OVX+30mIU hCG ($p = 0.07$) when compared to OVX+aCSF. Graphs represent average ±standard error of the mean. (* denotes $p < 0.05$ for 1-way ANOVA, SHAM+aCSF n = 13, OVX+aCSF n = 18, OVX+3mIU hCG n = 12, OVX+30mIU hCG n = 11). Abbreviations: OVX, ovariectomy; ICV, intracerebroventricular; aCSF, artificial cerebrospinal fluid.

Therefore, we analyzed LHR expression via Western blot with the cortex and hippocampus to see if LHR was still present (data not shown). As analyzed by 1-way ANOVA, there were no significant differences in LHR in the cortex ($F(3,18) = 0.910$, $p = 0.456$, $\eta^2 = 0.132$) or in the hippocampus ($F(3,17) = 0.350$, $p = 0.790$, $\eta^2 = 0.058$).

Because LHR activation can stimulate the phosphorylation of ERK, we analyzed ERK phosphorylation in the hippocampus (Fig. 4). There was a significant difference by 1-way ANOVA in phosphorylation of ERK ($F(3,16) = 6.752$, $p = 0.004$, $\eta^2 = 0.559$). Post hoc analysis revealed increased phosphorylated ERK in OVX+30mIU hCG as compared to the SHAM+aCSF ($p = 0.002$, d = 2.37), OVX+aCSF ($p = 0.001$, d = 2.09), and OVX+3mIU groups ($p = 0.024$, d = 1.33). Importantly, there was no significant difference by 1-way ANOVA in total ERK ($F(3,16) = 0.726$, $p = 0.552$, $\eta^2 = 0.127$).

With Western blotting of the hippocampus, we analyzed synaptophysin, a marker of synaptic vesicles, to address whether LHR activation in the CNS modulates synapses (Fig. 4). In the hippocampus, there was a significant difference by 1-way ANOVA in synaptophysin ($F(3,16) = 7.158$, $p = 0.003$, $\eta^2 = 0.573$), and post hoc analysis revealed higher expression of synaptophysin in OVX+3mIU hCG compared to SHAM+aCSF ($p = 4.0\text{x}10^{-4}$, d = 2.62), OVX+aCSF ($p = 0.008$, d = 1.88), and OVX+30mIU hCG ($p = 0.023$, d = 1.43).

Table 1
Serum LH of healthy cycling and ovariectomized mice-treated ICV with aCSF or hCG

Treatment	LH (ng/mL)
SHAM+aCSF	0.10±0.05
OVX+aCSF	3.70±0.45[a]
OVX+3mIU hCG	3.97±0.63[a]
OVX+30mIU hCG	4.91±0.85[a]

Key: LH, luteinizing hormone; aCSF, artificial cerebrospinal fluid; ICV, intracerebroventricular.
[a] Denotes significant ($p < 0.05$) from SHAM+aCSF.

3.5. *In vitro neurite outgrowth with hCG treatment*

To address whether LHR activation directly modulates neuronal morphology, we analyzed neurite outgrowth in hippocampal cultures treated with hCG (Fig. 5). One cell of the control group was a statistical outlier ($p < 0.05$) according to the Grubb's test and was therefore removed before final analysis. The treatment had significant effects by 1-way ANOVA on higher order neurites ($F(2,105) = 5.012$, $p = 0.008$, $\eta^2 = 0.087$), branch points ($F(2,105) = 5.123$, $p = 0.008$, $\eta^2 = 0.089$), and primary neurites ($F(2,105) = 4.510$, $p = 0.013$, $\eta^2 = 0.079$). Post hoc analysis shows that hCG increases the number of higher order neurites as compared to control ($p = 0.007$, d = 0.63) and deglycosylated ($p = 0.007$, d = 0.63). In addition, hCG increased the number of branch points as compared to the control ($p = 0.009$, d = 0.62) and deglycosylated hCG ($p = 0.005$, d = 0.65) groups. Interestingly, hCG treatment significantly increased the number of primary neurites compared to deglycosylated hCG-treated neurons ($p = 0.003$, d = 0.69).

4. Discussion

Despite surmounting evidence of LH involvement in cognitive deficits due to aging and neurodegenerative diseases, there is a shortage of studies on LH in the brain. Here, we demonstrated LH has a direct role in learning and memory through 3 novel findings. First, we observed a decrease in LH levels in several brain regions after OVX, indicating a loss of LH at a time point where cognitive deficits exist. Second, we rescued OVX-associated spatial memory deficits by administering an LH analogue, hCG, to the brain. Finally, hCG treatments increased ERK phosphorylation, synaptophysin expression, neurite outgrowth, and dendritic spine density.

To date, studies of memory impairments after OVX or menopause have focused on estrogens, which certainly have a role in cognitive decline and neuronal health. However, the critical period hypothesis and the healthy cell bias undercut HRT's use as therapeutic for age-related cognitive loss in females, and this shortcoming has, indeed, been demonstrated in animal models as well as humans (Blair et al., 2016; Brinton, 2005, 2008; Daniel and Bohacek, 2010; Daniel et al., 2006; Rapp et al., 2003; Sherwin, 2003; Zandi et al., 2002). The age-related changes of the HPG axis are critical to learning and memory deficits, but many studies have focused solely on E2 rather than the HPG axis (Gould et al., 1990; Luine and Rodriguez, 1994; Woolley et al., 1990; Woolley and McEwen, 1992). In this study focused on LH, we performed OVX in young adult animals because there is a robust effect on spatial memory (Heikkinen et al., 2004), and peripheral LH reaches a higher peak and is maintained for many months after OVX at a young age as compared to middle age (Gee et al., 1983). Our study was designed to elucidate the role of LH in the brain; thus, further study of brain

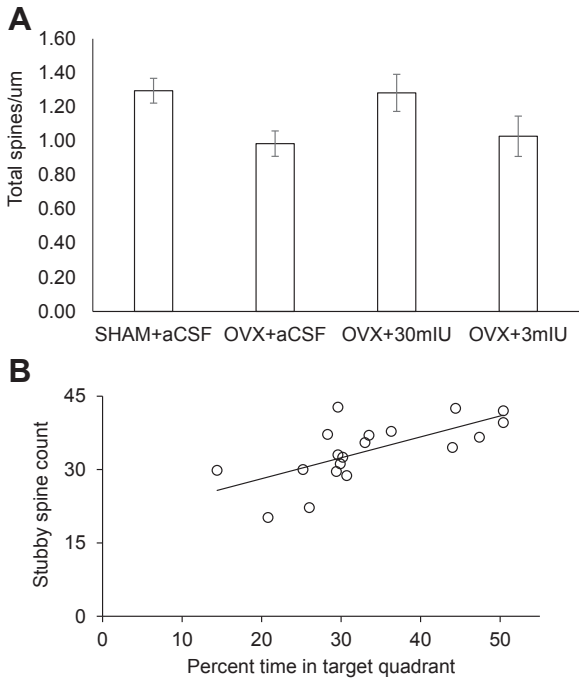

Fig. 3. Dendritic spine density of healthy cycling and ovariectomized mice treated ICV with aCSF or hCG. The Golgi method showed (A) significantly less stubby dendritic spines in retrosplenial cortex of OVX+aCSF as compared to SHAM+aCSF and OVX+30mIU hCG. Spine subtype counts revealed significantly less spines in OVX+aCSF as compared to SHAM+aCSF and OVX+30mIU hCG. (B) Percent time in the target quadrant of the Morris water maze correlates with stubby dendritic spine density. (* denotes $p < 0.05$ for 1-way ANOVA, SHAM+aCSF n = 6, OVX+aCSF n = 4, OVX+3mIU hCG n = 6, OVX+30mIU hCG n = 4). Abbreviations: OVX, ovariectomy; ICV, intracerebroventricular; aCSF, artificial cerebrospinal fluid.

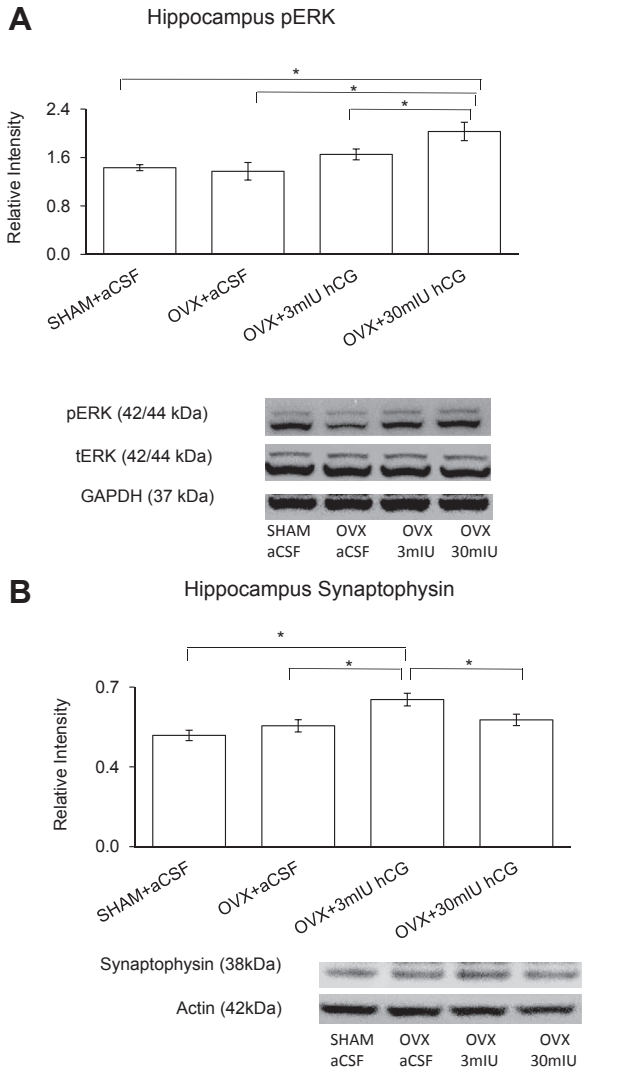

Fig. 4. pERK and synaptophysin of healthy cycling and ovariectomized mice-treated ICV with aCSF or hCG. There were significant differences in (A) the ratio of phosphorylated ERK to total ERK, and post hoc analysis revealed that OVX+30mIU had increased ERK phosphorylation as compared to SHAM+aCSF, OVX+aCSF, and OVX+3mIU hCG. There was no significant difference in the ratio of total ERK to GAPDH. There were significant differences in (B) synaptophysin to actin ratio with an increase in OVX+3mIU hCG as compared to SHAM+aCSF, OVX+aCSF, and OVX+30mIU hCG. Bar graphs represent average ±standard error of the mean. (* denotes $p < 0.05$ for 1-way ANOVA, SHAM+aCSF n = 5, OVX+aCSF n = 4, OVX+3mIU hCG n = 6, OVX+30mIU hCG n = 5). Abbreviations: OVX, ovariectomy; ICV, intracerebroventricular; aCSF, artificial cerebrospinal fluid; ERK, extracellular signal-related protein kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

LH as 1 component of the HPG axis throughout senescence is necessary. To this end, 1 hypothesis highlighting the HPG axis as an integrated whole throughout the aging process is that the hypothalamus loses responsiveness to estrogen during reproductive senescence (King et al., 1987). Thus, estrogen may no longer suppress LH, and this may cause a combined effect. Evidence of estrogen regulating brain LH levels is currently lacking but may be a fundamental aspect to the lack of estrogen benefits with a delay between OVX/menopause and treatment. Therefore, additional studies of brain LH, its relationship with estrogens, and its effect on cognition throughout the aging process are warranted.

Previous studies correlated increased peripheral LH with higher risk for AD (Butchart et al., 2013; Hogervorst et al., 2004; Short et al., 2001; Verdile et al., 2014), ultimately leading to the hypothesis that increased LH is detrimental to cognition (Burnham et al., 2017; Ziegler and Thornton, 2010); however, this hypothesis does not distinguish between peripheral LH and brain LH. On the contrary, we show elevated peripheral LH due to OVX coexists with decreased LH in several regions in the brain including the hippocampus, temporal cortex, and cingulate cortex, areas associated with learning and memory. In addition, our data suggest LH does not transport through the BBB in quantities that affect brain levels of LH because the elevated peripheral LH did not lead to elevated LH levels in the brain. Although we did not determine the mechanism underlying changes in brain LH, 1 hypothesis is a short feedback loop because LHR activation in the median eminence or the hypothalamus reduces pituitary LH levels (Corbin and Cohen, 1966; David et al., 1966; Hirono et al., 1972; Kawakami and Sawyer, 1959; Mores et al., 1996). Our data show hypothalamus LH levels are higher than other brain regions corroborating previous research (Emanuele et al., 1983), and it may be that hypothalamic LH is transported to different regions of the brain. Alternatively, we have

Table 2
Dendritic spine density subtypes of healthy cycling and ovariectomized mice-treated ICV with aCSF or hCG

Treatment	Stubby	Thin	Mushroom
SHAM+aCSF	0.882±0.07	0.326±0.03	0.085±0.008
OVX+aCSF	0.597±0.06[a,b]	0.325±0.05	0.065±0.004
OVX+3mIU hCG	0.720±0.05	0.326±0.05	0.084±0.004
OVX+30mIU hCG	0.888±0.07	0.302±0.05	0.093±0.005

Key: aCSF, artificial cerebrospinal fluid; ICV, intracerebroventricular.
[a] Denotes significant ($p < 0.05$) from SHAM+aCSF.
[b] Denotes significant ($p < 0.05$) from OVX+30mIU hCG.

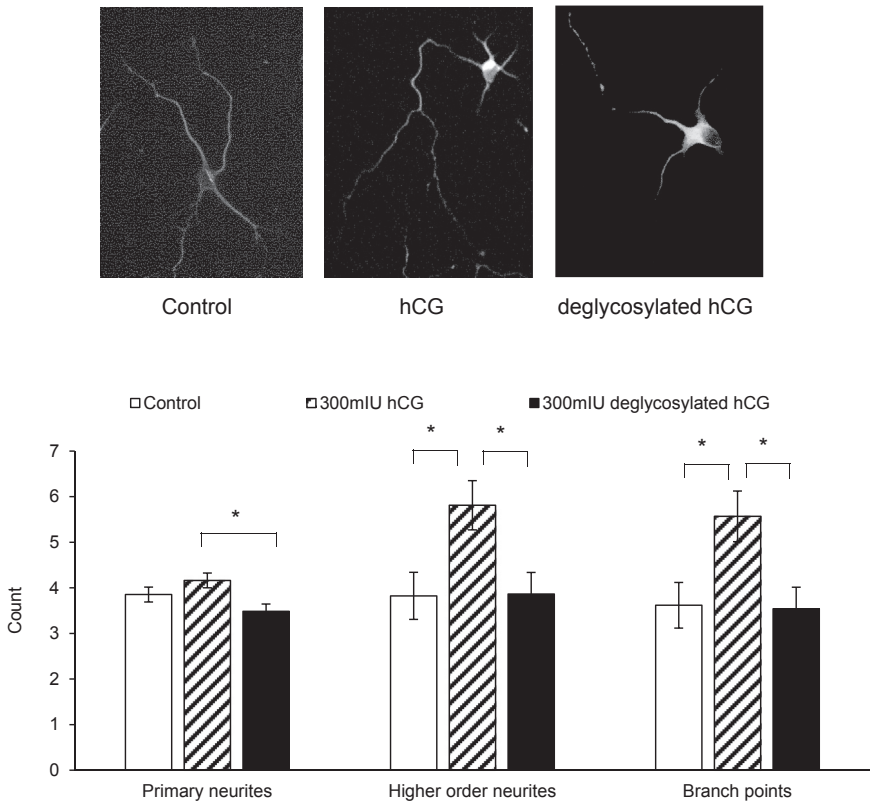

Fig. 5. Neuronal morphology in hippocampal cultures treated with hCG or deglycosylated hCG. Neurons were traced and primary neurites, higher order neurites, and branch points were counted. Treatment with hCG significantly increased both higher order neurites and branch points as compared to control and deglycosylated hCG. There was significantly fewer primary neurites in the deglycosylated hCG as compared to the hCG-treated group. Graphs represent average ±standard error of the mean. (* denotes $p < 0.05$ for 1-way ANOVA, Control n = 37, 300mIU hCG n = 34, 300mIU deglycosylated hCG n = 37, n = number of cells).

previously shown LH transcripts, both in the hippocampus and cortex, are lower in patients with AD as compared to controls (Palm et al., 2014); thus, LH may be endogenously translated in these memory-associated regions. The mechanism, however, for LH transcription and translation in the hippocampus has yet to be determined, but our data suggest some signal links the peripheral LH with brain LH production.

An LH analogue administered directly to the brain rescued spatial memory deficits despite the removal of estrogenic tissue (i.e., ovaries); thus suggesting an intact HPG axis or production of estrogen is not necessary for beneficial LH effects. This corroborates antide, Cetrorelix, and leuprolide acetate (LA) studies that lowered OVX-associated increased peripheral LH and rescued spatial memory deficits (Blair et al., 2016; Bryan et al., 2010; Palm et al., 2014; Telegdy et al., 2009; Ziegler and Thornton, 2010). Importantly, antide administered to the brain does not rescue spatial memory, possibly because the GnRHR modulators derive benefits from a decrease in peripheral LH rather than modulating GnRHR directly in the hippocampus (Burnham et al., 2017); however, our ICV hCG treatment successfully rescued spatial memory, whereas peripheral LH remained elevated. Brain LH is possibly the crux of these studies and modulating peripheral LH may be efficacious through its ability to modulate brain LH. As such, our previous study demonstrated that LA not only downregulated peripheral LH but also increased brain LH (Palm et al., 2014). Furthermore, unlike estrogen treatment, LA rescues spatial memory beyond the critical period in the mouse OVX model (Blair et al., 2016) as well as in female patients with AD (Bowen et al., 2015). Thus, together these studies accumulate evidence for brain LH as a target for therapeutics.

Decreased brain LH after OVX and rescued spatial memory by ICV hCG are evidence against 1 hypothesis in the literature (Burnham et al., 2017; Ziegler and Thornton, 2010); however, not all data support our hypothesis; LH is beneficial to cognition. ICV hCG administration showed no change in spatial memory in rats (Lukacs et al., 1995), and intrahippocampal hCG in OVX rats abolishes E2 benefits (Burnham et al., 2017), but the doses used in these 2 studies were 300-fold higher than the dose used here. In addition, high doses of hCG/LH can downregulate LHR (Hu et al., 1990; Peegel et al., 1994; Segaloff et al., 1990). Thus, these behavioral changes may be due to a loss of LHR in these previous studies. Importantly, we showed our doses of hCG did not downregulate LHR in vivo, and our previous study suggests our in vitro dose increases LHR (Palm et al., 2014).

In addition to rescued spatial memory, hCG increased neurite outgrowth and rescued stubby dendritic spine density. Therefore, hCG may rescue spatial memory by increasing structural plasticity. This agrees with our previous study of LA showing decreased peripheral LH rescued OVX-associated loss of dendritic spine density. In addition, LA was effective both when treatment was administered the same day as OVX and 4 months after OVX, a time point where E2 had no effect (Blair et al., 2016). We measured brain LH in a previous study and LA downregulated peripheral LH but increased brain LH (Palm et al., 2014). This ability of GnRHR modulators to decrease peripheral LH while increase brain LH may explain the increase in dendritic spines seen with GnRHR modulators.

The ERK pathway is involved in learning and memory, including underlying mechanisms such as synaptic plasticity and spine density (Blum et al., 1999; English and Sweatt, 1997; Goldin and Segal, 2003; Hebert and Dash, 2002; Selcher et al., 1999). In addition, ERK

activation is necessary for LHR stimulated differentiation of PC12 cells (Meng et al., 2007). Thus, our data of increased ERK phosphorylation due to hCG treatment corroborate previous findings. Together, with the increased dendritic spine density, these data provide a potential molecular mechanism for LHR in the brain.

Synaptophysin, a synaptic vesicle protein, is a pseudo-marker of the number of synapses. As such, it decreases with age (Smith et al., 2000) and AD (Honer, 2003; Masliah et al., 2001). Dendritic spines decrease due to OVX (Woolley and McEwen, 1992); thus the lack of a significant reduction in synaptophysin levels due to OVX was unexpected. On the other hand, LHR activation benefits neuronal morphology (Al-Hader et al., 1997a; Meng et al., 2007), and we did show synaptophysin expression was increased with ICV hCG. This suggests an increased strength or number of synapses, in agreement with the dendritic spine data. Furthermore, synaptophysin expression is regulated by a noncanonical notch signaling pathway (Hayashi et al., 2016), and Notch1 is involved in synaptic plasticity and short-term memory (Alberi et al., 2011). Thus, LHR may drive notch-related signaling to garner learning and memory benefits.

LH and LHR are found in a variety of brain regions (Apaja et al., 2004; Emanuele et al., 1983; Lei et al., 1993), and we observed decreases in LH in several regions of the brain; however, our study was not designed to determine which brain regions are responsible for the rescue of spatial memory. Hippocampus, temporal cortex, and cingulate cortex may all be responsible for the spatial memory changes we observed, but the presence of LHR in several other regions suggests LH effects may extend beyond spatial memory. As such, LH may be involved in sleep (Toth et al., 1994), neurogenesis (Mak et al., 2007), courtship behavior (Yang et al., 2007), or general activity (Lukacs et al., 1995), but these aspects need further investigation.

Whether ICV hCG rescue of spatial memory was gained exclusively from the neuronal population is unknown, but our in vitro neurite outgrowth experiment showed neurons undergo additional branching in the presence of hCG. This corroborates previous work in PC12 cells overexpressing LHR that had increased neurite outgrowth and differentiation because of hCG treatment (Meng et al., 2007) as well as LHR activation differentiates stem cells (Gallego et al., 2010). In addition, previous primary neuron studies showed hCG protects from excitotoxicity (Movsas et al., 2017) and stimulates signaling (Palm et al., 2014). Furthermore, we previously showed LH packaged in vesicles of secretory vesicle size (Blair et al., 2015), but the neuronal population is diverse and the cell type is unknown. Also, we cannot exclude that our treatment may have also stimulated LHR on non-neuronal cells such as glia (Al-Hader et al., 1997b).

5. Conclusion

Overall our data suggest brain LH has a role in learning and memory as well as underlying mechanisms such as structural plasticity. Importantly, we show brain LH is decreased after OVX and hCG administration to the brain after OVX rescues spatial memory and dendritic spine density despite peripheral LH levels remaining elevated. Even though there is no known mechanism for linking peripheral LH and brain LH, human patients with AD and OVX rodent models have decreased brain LH at times when peripheral LH is elevated (Palm et al., 2014). Rescued spatial memory and neuronal morphology by direct administration of hCG to the brain is possibly explained by LHR signaling mechanisms and remains a goal for future studies. Altogether, our current data highlight the potential for therapeutics that ultimately upregulate brain LH.

Disclosure

The authors have no actual or potential conflicts of interest.

Acknowledgments

This work was supported by the National Institute on Aging (1RF1-AG054654-01). The University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core is supported by the Eunice Kennedy Shriver NICHD/NIH (NCTRI Grant P50-HD28934).

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