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Luteinizing hormone downregulation but not estrogen replacement improves ovariectomy-associated cognition and spine density loss independently of treatment onset timing

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Abstract

Age-related changes in reproductive hormone levels are a well-known risk factor for the development of cognitive dysfunction and dementia in women. We and others have shown an important contribution of gonadotropins in this process. Lowering serum gonadotropin levels is able to rescue cognitive function in Alzheimer's disease and menopause models, but whether this is time-dependent and the exact mechanism through which gonadotropins regulate cognitive function is unknown. We show that pharmacologically lowering serum levels of luteinizing hormone lead to cognitive improvement immediately after ovariectomy and with a 4 month interval after ovariectomy, when the benefits of 17 β -estradiol are known to disappear in rodents. Importantly, we show that these improvements are associated with spine density changes at both time points. These findings suggest a role of luteinizing hormone in learning and memory and neuroplasticity processes as well as provide an alternative therapeutic strategy of menopause associated cognitive loss.

Keywords

luteinizing hormone; spine density; ovariectomy; cognition; estrogen; aging; timing; menopause

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1. Introduction

Hormonal changes due to aging, such as the loss of estrogens and the corresponding increase in serum luteinizing hormone (LH), are associated with cognitive decline. Historically, emphasis has been placed on understanding the mechanism underlying CNS dysfunction after estrogen loss at menopause. This is based on the recognized effects of 17 β -estradiol (E2) on neuronal structure and function (Woolley and McEwen, 1992) and dysfunction after ovariectomy (OVX) at a neuronal (Woolley and McEwen, 1992; Waters et al. 2009; Tanapat et al., 1999) and functional level (reviewed McEwen et al. 2012). E2 treatment after OVX rescues cognitive function and associated mechanisms in mice and rats (Heikkinen et al., 2004, McLaughlin et al., 2008, Daniel et al., 2006). Additionally, E2 has benefits in humans, such as improving cognition in younger women who have undergone oophorectomy (Sherwin, 1988). However, introducing a long delay between OVX and treatment onset renders E2 ineffective in rodents at restoring cognition (Gibbs 2000, Heikkinen et al., 2004, Daniel et al., 2006) and rescuing spine density (McLaughlin et al. 2008). The differential effects of E2 have led to the critical period hypothesis (Daniel and Bohacek 2010) that E2 replacement has an ideal time frame of effectiveness in rodents. Associated with this theory is the healthy cell bias hypothesis which states that E2 is beneficial during health, but is detrimental in the diseased or stressed state, such as that of aging (Brinton 2008). Unfortunately, the underlying mechanisms remain unknown.

Independent of the ability of estrogens to regulate cognitive and neuronal function, increasing data support a role for LH on cognition. In this regard, serum LH levels are higher in Alzheimer's disease (AD) patients (Short et al. 2001; Butchart et al., 2012), are associated with increased AD pathology in men (Verdile et al. 2008; Verdile et al., 2014) and cognitive dysfunction in older women (Rodrigues et al. 2008). In rodents, pharmacologically lowering LH levels with leuprolide acetate, a GnRHR super-agonist, is as effective as E2 replacement at improving cognitive function in ovariectomized mice (Bryan et al., 2010) as well as in AD models (Casadesus et al., 2006, Palm et al. 2014). Benefits of reducing serum LH levels on cognition have also been shown using the classic GnRHR antagonists such as antide in male and female rats (Ziegler et al. 2010, McConnell et al. 2012) and cetrorelix in rats and mice (Telegdy et al., 2009; Telegdy et al., 2010). Given the different mechanism of action of these drugs, but common end result (i.e. lowering LH levels), this suggests that peripheral LH rather than GnRHR signaling is associated with cognitive benefits. Importantly, a clinical trial in female AD patients revealed that in a subgroup taking acetylcholinesterase inhibitors, leuprolide acetate slows the progression of AD (Bowen et al., 2015).

In order to address whether pharmacologically lowering serum LH holds similar time dependent effects to that of E2 replacement on cognition and dendritic spine density as shown in previous studies in rodents (Gibbs 2000, Heikkinen et al., 2004, Daniel et al., 2006, McLaughlin et al. 2008) we determined: 1) whether leuprolide acetate rescues cognition after OVX both where E2 is effective (immediately after OVX) and ineffective (4 months after OVX) in middle-aged post-partus female mice and 2) whether changes in cognition observed in leuprolide acetate treated animals are associated with changes in spine density morphology as related to those observed for E2 replacement at both time points.

2. Methods

2.1 Animals and experimental timeline

C57Bl/6J retired breeders, 9 months of age, were purchased from Jackson Laboratory (Bar Harbor, Maine). Animals were group-housed in a humidity and temperature controlled room with a 12-hour light:dark cycle starting at 6AM. Throughout the duration of the study all animals were supplied with food and water *ad libitum*. Animals were allowed to acclimate to the animal facility for one week before any experimental manipulations. All experimental procedures and animal care was done in accordance with the Institutional Animal Care and Use Committee of Case Western Reserve University.

2.2 Experimental design

Animals were ovariectomized at 9 months of age and were either treated right after OVX or treated 4 months after OVX. All animals were treated for 8 consecutive weeks. During the last week of treatment, spatial learning and memory was assessed with the Morris water maze task, which occurred at 11 months of age for the no delay cohort and 15 months of age for the 4 month delay cohort (Figure 1).

2.3 Ovariectomy and pump implant

Mice were deeply anesthetized and ovaries were either removed or exposed and reinserted (SHAM) bilaterally through a single incision. The no delay cohort was subcutaneously implanted with either physiological saline, E2 (1.1ng 17 β -estradiol/day) or leuprolide acetate (3.6 μ g leuprolide acetate/day) in an osmotic pump (Alzet 1004, Durect Corporation, Cupertino, California) on the day of OVX (no delay) or after a 4 month delay between OVX and treatment onset (4 month treatment delay). It is known that serum E2 levels peak at 66pg/ml during estrous (Wood et al., 2007; Fata et al., 2001), and we have previously shown that this treatment regimen produces physiological levels of E2 in the bloodstream (12–85pg/ml, Palm et al., 2014). Both no delay and 4 month treatment delay cohorts underwent pump replacements monthly for the duration of the study. All procedures were performed in an aseptic environment.

2.4 Morris water maze

Mice were habituated to handling daily for two weeks prior to training on the Morris water maze (MWM) task. A 110 cm diameter pool in the center of a room with visual cues on each wall was filled with white opaque water maintained at 23°C and a submerged 10.5cm diameter platform was covered by 0.5cm of water. On the first day each animal was placed in the water and guided to the platform in order to acclimate the animal to swimming and the apparatus. During training, animals were placed gently into the water facing the wall of the pool from each quadrant and allowed to swim either until they found the platform or until 60 seconds elapsed, in which case they were gently guided to the platform and given 15 seconds on it prior to the start of the next trial. After 4 consecutive trials animals were dried and placed in the home cage on a heating pad until the start of the next session. Each animal was trained in 2 sessions of 4 trials each per day for 4 days. On the last trial of day 4 the platform was removed for a probe trial. During this trial, time spent in the target quadrant

was recorded for one 60 second trial to determine spatial strategy use and hippocampal memory consolidation. All trials were tracked and analyzed using Ethovision 7.0 (Noldus, Leesburg, Virginia). Animals unable to swim for the entirety of the behavioral testing were excluded from analyses.

2.5 Serum Collection and Tissue Processing

On conclusion of behavioral testing, all animals were deeply anesthetized, weighed, and blood was collected by either cardiac puncture or via the orbital sinus. Blood was allowed to coagulate and after centrifugation the serum was decanted and stored at -20°C until it was shipped for analysis. Serum LH assays were performed by the Ligand Assay and Analysis Core at the University of Virginia. After blood collection, half of the animals were transcardially perfused with saline, then 4% paraformaldehyde before the brains were removed and bisected. One hemisphere was post-fixed in 4% paraformaldehyde for an additional hour, incubated in 30% sucrose overnight and then stored at -20°C in 30% glycerol 30% sucrose 0.2% NaN_3 . The other hemisphere was post-fixed in 4% paraformaldehyde for 4 hours, incubated in 30% sucrose overnight, and then frozen in tissue freezing medium for storage at -80°C . The uterine weight was taken for all animals at the end of the study in order to verify OVX status.

2.6 Spine Density

Hemispheres stored in 30% glycerol 30% sucrose 0.2% NaN_3 were thawed, cut coronally at $250\mu\text{m}$ on a vibratome, and placed in PBS in a 12-well plate. A Helios gene gun was used with $1.6\mu\text{m}$ gold particles to deliver lipophilic dialkylcarbocyanine (Staffend and Meisel, 2011) at 200 psi. The apical dendrite of pyramidal neurons residing in retrosplenial cortex and cingulate cortex layer II/III was imaged with a Zeiss LSM 510 Meta equipped with a motorized stage. Three dimensional reconstructions were made from z-stacks in Neuron studio (CNIC, Mount Sinai School of Medicine) and were used to trace apical dendrites, then perform Scholl analysis to classify and quantify spines $5-15\mu\text{m}$ from the soma (Rodriguez et al., 2008). 3–5 mice were used for each treatment group and multiple neurons were quantified for each animal.

2.7 Statistic Analysis

All statistical analyses were performed with SPSS 17.0 (SPSS Inc., Chicago, Illinois). Statistical outliers were detected by using the Grubb's outlier test. For the MWM task a repeated-measures ANOVA was used with the latency to the platform for training days as the within group and the treatment as between groups. The area under the curve (AUC) for latency during training was used to illustrate treatment effects (Heneka et al. 2013; D'Agostino et al. 2012; Walter et al. 2011). Duration in the target quadrant and spine density data were analyzed with a one-way ANOVA using *post-hoc* analysis where appropriate. Effect size is described by Cohen's d for pairwise comparisons and eta squared for all ANOVA statistics where $\eta^2 = \text{SS}_{\text{effect}} / \text{SS}_{\text{total}}$.

3. Results

As expected a high serum LH level confirmed OVX status and a reduced serum LH level confirmed E2 and leuprolide acetate efficacy (Table 1). A two-way ANOVA was used to analyze serum LH levels and showed that there was no interaction between timing and treatment ($F_{(3,73)}=0.562$; $p>0.05$; $\eta^2=0.004$), but a significant difference was found with treatment ($F_{(3,73)}=101.89$; $p<0.001$; $\eta^2=0.81$). Serum LH levels were increased by OVX (no delay: $p<0.01$; $d=2.98$, 4M delay: $p<0.01$; $d=3.42$) and rescued to basal levels with leuprolide acetate (no delay: $p>0.05$; $d=-0.81$, 4M delay: $p>0.05$; $d=-1.18$) and E2 treatments (no delay: $p>0.05$; $d=-0.95$, 4M delay: $p>0.05$; $d=-1.25$) compared to SHAM in both no delay ($F_{(3,35)}=42.91$; $p<0.001$; $\eta^2=0.80$) and delay ($F_{(3,37)}=60.21$; $p<0.001$; $\eta^2=0.84$) cohorts.

3.1 Leuprolide acetate rescues spatial memory after OVX regardless of onset of treatment

In the no delay cohort a repeated-measures ANOVA for escape latency, the time to find the hidden platform, showed a significant difference for the within subject factor (day of training) that illustrates the animals were successfully trained ($F_{(3,108)}=60.46$; $p<0.001$; $\eta^2=0.58$; Figure 2A). There was also a significant interaction between day and treatment ($F_{(9,108)}=2.72$; $p<0.01$; $\eta^2=0.08$). *Post-hoc* analysis indicated that ovariectomized animals took longer to locate the platform than SHAM ($p<0.001$; $d=-1.71$) and that this deficit was rescued by both E2 ($p<0.01$; $d=-1.97$) and leuprolide acetate ($p<0.05$; $d=-0.95$) treatments. Area under the curve (AUC) for latencies, which represents difference between treatment groups, is also shown (Figure 2B). In the probe trial there was a difference between treatment groups as shown by a one-way ANOVA ($F_{(3,39)}=3.46$; $p<0.05$; $\eta^2=0.22$). *Post-hoc* analysis showed that ovariectomized animals spent less time in the target quadrant than SHAM ($p<0.05$; $d=1.62$) and that this aspect was rescued by E2 ($p<0.05$; $d=1.01$) and leuprolide acetate ($p<0.05$; $d=1.59$) treatments (Figure 2C).

Morris water maze performance was also analyzed in the 4 month treatment delay cohort. Repeated-measures ANOVA analysis revealed a significant difference between treatment groups for latency to platform during training ($F_{(3,30)}=4.46$; $p<0.05$; $\eta^2=0.30$). Successful training was indicated by a significant difference in the within subjects factor (day of training) ($F_{(3,90)}=39.38$; $p<0.001$; $\eta^2=0.52$; Figure 3A). AUC for latency to the platform is shown to illustrate between group differences (Figure 3B). *Post-hoc* analysis revealed that the leuprolide acetate group took less time to locate the platform than any other group (SHAM: $p<0.05$; $d=1.04$, OVX: $p<0.05$; $d=1.37$, E2: $p<0.05$; $d=2.27$), but there was no difference between OVX and SHAM ($p>0.05$; $d=-0.06$). For the probe trial, one-way ANOVA analysis revealed a difference between treatment groups ($F_{(3,34)}=4.17$; $p<0.05$; $\eta^2=0.29$; Figure 3C). *Post-hoc* analysis demonstrated that the leuprolide acetate-treated mice spent more time in the target quadrant than any other group (SHAM: $p<0.05$; $d=-1.16$, OVX: $p<0.05$; $d=-0.91$, E2: $p<0.05$; $d=-1.27$, Figure 3C).

3.2 LA treatment rescues spine density after OVX

After Morris water maze testing animals were sacrificed and tissue was processed and diolistically labeled with a gene gun to evaluate changes in spine morphology of the apical

dendrite in cortical layer II/III pyramidal neurons. A two-way ANOVA was used to analyze the effect of treatment group and treatment onset on spine density. There was no significant interaction between treatment group and treatment onset for spine density ($F_{(3,96)}=0.518$; $p>0.05$; $\eta^2=0.01$). However, there was a significant main effect of treatment onset between no delay and 4-month delay treatment ($F_{(1,96)}=18.79$; $p<0.001$; $\eta^2=0.13$) and a significant main effect of treatment group on spine density ($F_{(3,96)}=6.56$; $p<0.001$; $\eta^2=0.14$). For both the no delay (SHAM: $n=17$ cells/5 animals; OVX+SAL: $n=15$ cells/4 animals; OVX+LA: $n=15$ cells/4 animals; OVX+E2: $n=12$ cells/3 animals; $F_{(3,58)}=2.99$; $p<0.05$; $\eta^2=0.14$) and 4-month delay treatment (SHAM: $n=45$ cells/5 animals; OVX+SAL: $n=27$ cells/3 animals; OVX+LA: $n=34$ cells/4 animals; OVX+E2: $n=35$ cells/4 animals; $F_{(3,140)}=6.57$; $p<0.001$; $\eta^2=0.12$) cohorts the OVX group displayed fewer total spines compared to the SHAM group (no delay: $p<0.05$; $d=0.94$, 4M delay: $p<0.05$; $d=0.73$, Figure 4). E2 replacement was not able to significantly increase total spine number in neither the no delay nor the 4-month delay interventions (no delay: $p>0.05$; $d=0.80$, 4M delay: $p>0.05$; $d=0.46$, Figure 4A). However, lowering luteinizing hormone levels with leuprolide acetate was able to rescue total spine densities to SHAM levels as compared to OVX (no delay: $p<0.05$; $d=0.89$, 4M delay: $p<0.001$; $d=0.96$, Figure 4A). Importantly, we also identified differences in spine type across groups. For example, in the no delay cohort the SHAM and leuprolide acetate groups showed a significantly increased number of thin spines compared OVX ($F_{(3,58)}=6.15$; $p<0.05$; $\eta^2=0.15$, SHAM: $p<0.05$; $d=0.91$, LA: $p<0.01$; $d=0.92$, Figure 4C). In the 4-month delay cohort an increase in the number of mushroom spines was observed ($F_{(3,140)}=3.95$; $p<0.05$; $\eta^2=0.08$) with leuprolide acetate treatment compared to both the OVX and E2 groups (OVX: $p<0.05$; $d=-0.75$, E2: $p<0.05$; $d=-0.53$, Figure 4D).

4. Discussion

We compared the effects of pharmacologically lowering serum LH and E2 replacement on cognition and spine density in middle-aged ovariectomized female mice when treatments were administered immediately after OVX (no delay) or after an interval of 4 months between OVX and treatment onset (4 month delay). Our data demonstrate that peripheral LH is a key player in driving ovariectomy-related cognitive dysfunction and spine density loss. Importantly, we have also determined that unlike E2 replacement, lowering peripheral LH is effective at improving cognition and spine density beyond the “critical” period of E2 replacement effectiveness in rodents.

Importantly, we show that ovariectomized animals treated with leuprolide acetate to lower peripheral levels of LH show improvements in spatial memory both when treatment onset is immediately after or 4 months after OVX. In contrast, E2 replacement was only effective at improving cognition when administered immediately after OVX. While the mechanisms underlying these differences is unknown, one explanation for the differences between treatments may lie in the fact that E2 treatment is beneficial while neuronal health remains stable, but E2 treatment becomes detrimental when neurons are in poor health (Brinton, 2008).

Interestingly, in our 4 month delay cohort we did not identify significant differences in cognitive function between SHAM and OVX or E2 treated animals, but did observe that

leuprolide acetate treated animals showed significant cognitive improvements. This suggests an independent mechanism of action of LH on cognition. The fact that SHAM control animals were not different from OVX or E2 in the 4 month delay cohort is not surprising since this cohort was tested at 15 months of age, an age when deficits in the MWM task are prevalent in normal animals (de Fiebre et al., 2006). However, our data highlights the fact that lowering serum LH in aged animals, despite of their E2 status is beneficial and will need to be explored in depth in future studies.

In addition to cognitive benefits, leuprolide acetate treatment maintained the total number of spines, a marker of synaptic plasticity, at SHAM levels in both no delay and 4 month treatment delay cohorts. Dendritic spines have a low rate of turnover in adults, but morphology changes rapidly and spine plasticity is strongly associated with learning and memory (Gutierrez and Davies, 2011). Therefore, our data suggest that a potential mechanism underlying improvements in cognition by lowering peripheral LH levels involves its ability to regulate spine density.

Contrary to previous studies showing increases in spine density with E2 treatment (Woolley and McEwen, 1992; Gould et al., 1990; Bailey et al., 2011), we did not observe such benefits. While E2 did show a trend toward increasing OVX-associated loss of spine density when administered immediately after OVX surgery, these differences were not statistically significant. These findings may be explained by differences in age, parturition status of the females, treatment length, and/or different methods of delivery of E2 across studies. It is also possible that because the animals were 11 months of age when scarified, based on the healthy-bias hypothesis of E2 (Brinton et al., 2008) that the effects of estrogen were mitigated by the aging, more stressful, neuronal environment. However, given that lowering of peripheral LH to SHAM levels increased spine density like E2 has been shown to do, our data suggest that some of the E2 effects may be secondary to lowering OVX-induced rises in peripheral LH.

Alternatively, it is also possible that OVX-related loss of E2 and rises in peripheral LH regulate neuronal plasticity in parallel but independently both via the regulation of spine density and other signals. This latter aspect is highlighted by the fact that while SHAM operated animals showed significantly higher levels of spines compared to OVX and E2 animals, SHAM operated animals did not show differences in cognitive function when compared to OVX or E2 animals in the 4 month delay treatment cohort. Previous published data from our group demonstrates that lowering LH with leuprolide acetate alters signaling molecules associated with development and stabilization of spines (Palm et al., 2014) and leads to activation of signaling cascades associated with long term potentiation (Bryan et al., 2010). Therefore it is plausible that additional mechanisms are involved in the ability of leuprolide acetate to improve cognition above and beyond the performance observed in SHAM treated animals in the 4-month delay cohort.

In addition to changes in total spine density, our data also show that restoring OVX-related increases in serum LH levels also alters spine type. Mature mushroom spines have large postsynaptic densities and are generally viewed as stable, while the plastic, thin spines are less stable due to their dynamic nature (Bailey et al., 2011). It is thought that the conversion

from thin spines to mushroom spines can occur with the induction of long-term potentiation and long-term depression. Our data revealed that depending on treatment onset, different types of spines were more prone to being altered. For example, in the no delay cohort, when animals were younger, lowering peripheral LH rescued OVX-related loss in thin spines to SHAM levels, indicative of changes in plasticity, but not mushroom spines. These findings are similar to those in an aging study where thin and stubby spines were decreased due to aging, and were affected by stress (Bloss et al., 2011). Though mushroom spines are associated with stability of synapses, an increase in spine head diameter has been observed with aging, despite the loss of thin and stubby spines (Bloss et al., 2011). In our data leuprolide acetate treatment increased the number of mushroom spines in the older animals even though no decrease was observed in the OVX group when compared to control.

Previously published data support the role of LH in cognition. For example, we and others have demonstrated that pharmacologically lowering OVX-related increases in serum LH improves cognitive function in rodents (Bowen et al., 2004; Bryan et al., 2010; Casadesus et al., 2006; Kovacs et al., 2001; Palm et al., 2014; Telegdy et al., 2009; Ziegler and Thornton, 2010). While a non-specific pharmacological effect of leuprolide acetate cannot be ruled out, positive effects on cognition achieved by lowering peripheral LH levels have been attained using different types of drugs. Both competitive antagonists like antide or cetrorelix (Kovacs et al., 2001; Telegdy et al., 2009; Ziegler and Thornton, 2010) as well as leuprolide acetate (Bryan et al., 2010; Casadesus et al., 2006; Palm et al., 2014), a super agonist that drives its effects by downregulating rather than blocking the GnRHR, improve OVX-associated cognitive deficits. As such, while these drugs work differently at the GnRHR, all have a common end result, to reduce peripheral LH levels. Together these data suggest that it is the reduction of peripheral LH rather than non-specific effect of leuprolide that drives benefits on cognition and plasticity. Additionally, we provide supplemental data (figure 1 supplemental materials) showing that levels of hypothalamic GnRH mRNA are similar in OVX and leuprolide-treated animal suggesting that changes in GnRH processing is also an unlikely factor to drive the observed effects of leuprolide. Lastly, studies show that peripheral injections of an LH analogue (hCG) attenuate E2 associated rescues in spatial memory (Barron et al., 2010; Berry et al., 2008). Given that hCG, like LH, signals through the LH receptor these data suggest that peripheral activation of the LH receptor impact cognition negatively despite of E2 status; an aspect that has been shown for conditions associated with high LH levels such as polycystic ovarian syndrome (Barnard et al., 2007; Yen et al., 1970).

The mechanism through which lowering peripheral levels of LH restores cognition and spine density is unknown, but evidence from our lab supports a role for LH signaling in the brain. We have shown that AD patients have lower levels of LH mRNA in both the hippocampus and cortex (Palm et al., 2014) despite having higher plasma levels compared to controls (Short et al., 2001; Butchart et al., 2012). In the rodent we have shown that ovariectomy-associated increases in serum LH are associated with reductions in LH immunoreactivity in cognition-associated areas, and that these LH levels are restored with leuprolide acetate treatment (Palm et al., 2014). Similarly, by observing LH levels in rats throughout the estrous cycle it has been shown that hypothalamic LH decreases during proestrus, when pituitary and serum LH levels are high (Emanuele et al., 1981). Due to the

presence of LH mRNA (Palm et al., 2014), the presence of functional LH protein in the brain (Blair et al., 2015; Palm et al., 2014; Emanuel et al., 1983; Hostetter et al., 1981) and the inverted relationship between pituitary/serum LH levels and brain LH levels (Palm et al., 2014; Emanuele et al., 1981), we believe that LH is made in the brain. It is also known that LH receptor is localized to regions of the brain important for spatial reasoning, such as the hippocampus (Apaja et al., 2004). Additionally, activating LH receptor increases differentiation in PC12 cells, a neuron-like cell (Meng et al., 2007), and in vivo LH receptor activation affects courtship songs in *Xenopus laevis* (Yang et al., 2007) and activity and neurogenesis in rodents (Lukacs et al., 1995; Mak et al., 2007). Therefore, one potential mechanism is that serum LH levels regulate, through some yet to be discovered feedback mechanism, the production of brain-derived LH. This, in turn, drives LH receptor signaling in the brain, which regulates cognition and plasticity (reviewed Blair et al., 2015). One such mechanism could be the short-loop feedback mechanism between the pituitary and hypothalamus (Emanuele et al., 1981; Conway and McCann, 1990). In fact, projections from the hypothalamus may be supplying LH as a neurotransmitter in higher quantities when the peripheral levels of LH are decreased, but future studies are necessary to test this hypothesis.

Taken together our data suggest that LH associated mechanisms are key in the regulation of learning and memory and underlying mechanisms of this function such as dendritic spine density. Importantly, lowering OVX-associated rises in serum LH, unlike E2 replacement, is effective regardless of timing of treatment onset in rodents. Whether the benefits of lowering LH are dependent or independent of estrogens remains unclear and a goal for future studies. However, at the very least these data clearly demonstrate that lowering peripheral LH levels induced by loss of estrogens is more effective than E2 replacement in the aged rodent brain. Furthermore, our data also suggests that additional mechanisms, beyond spine density, may be influencing the benefits on cognition that are observed after lowering levels of serum LH. Though the root mechanisms are yet to be discovered, these data and the recently published findings by Bowen et al., 2015, which show leuprolide acetate treatment benefits female patients with AD, highlight the potential for leuprolide acetate as a therapeutic agent and the need for further studies characterizing the role of LH in cognition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Leuprolide acetate and E2 rescue deficits on the Morris water maze task when treated immediately after OVX
- At a time point after OVX where E2 is not beneficial, leuprolide acetate improves spatial learning and memory
- OVX-associated loss of spines density on dendritic outgrowths is rescued by leuprolide acetate

Figure 1. Schematic diagram for OVX, treatments and Morris water maze

The no delay cohort was sham operated or ovariectomized at 9 months of age and immediately treated with saline, E2 or leuprolide acetate. After 8 weeks of treatment the no delay cohort was trained on the Morris water maze behavior paradigm. Animals were sacrificed on the last day of the Morris water maze. The delay cohort was sham operated or ovariectomized at 9 months of age, but no treatments were given on the day of surgery. Four months after surgery, the delay cohort began treatment with saline, E2 or leuprolide acetate. After 8 weeks of treatment the delay cohort underwent Morris water maze testing and on the final day the animals were sacrificed.

Figure 2. Leuprolide acetate rescues deficits on the Morris water maze task with no delay of treatment after OVX

In the no delay cohort, where treatment began on the day of OVX, OVX-associated cognitive dysfunction in the Morris water maze was rescued by 8 weeks of treatment with leuprolide acetate or E2. A: Animals were successfully trained over a four day period. B: Bar graphs represent area under the curve (AUC) for accumulative latency to the platform during training. Leuprolide acetate and E2 had a lower latency to the platform (repeated measure ANOVA). C: The duration the animal spent in the target quadrant was used to analyze the sixty second probe trial, where the platform was removed. E2 and leuprolide acetate treated animals spent a higher percentage of time in the target quadrant as compared to the ovariectomized group (one-way ANOVA). Results are expressed as mean $\pm$ sem. Significant difference ($p < 0.05$) from SHAM+SAL (a), from OVX+SAL (b), from OVX+LA (c) and from OVX+E2 (d)

SHAM+SAL, sham ovariectomy treated with saline; OVX+SAL, ovariectomy treated with saline; OVX+E2, ovariectomy treated with 17 β -estradiol; OVX+LA, ovariectomy treated with leuprolide acetate

Figure 3. Leuprolide acetate rescues spatial learning and memory with a delay of 4 months after ovariectomy

The 4 month delay cohort, who had a 4 month delay between OVX and treatment, showed improvements in the Morris water maze task only in the group treated for 8 weeks with leuprolide acetate. A: Similarly to the no delay cohort, animals were trained for 4 days. B: The leuprolide acetate group had a lower latency to the platform during training (repeated measures ANOVA). The bar graph represents the AUC for the latency to the platform for the training trials. C: The duration in the target quadrant during the probe trial showed a significant difference between treatment groups (one-way ANOVA), with the leuprolide acetate treatment spending more time in the target quadrant than any other group. Results are expressed as mean $\pm$ sem. Significant difference ($p < 0.05$) from SHAM+SAL (a), from OVX+SAL (b), from OVX+E2 (c) and from OVX+LA (d) SHAM+SAL, sham ovariectomy treated with saline; OVX+SAL, ovariectomy treated with saline; OVX+E2, ovariectomy treated with 17β -estradiol; OVX+LA, ovariectomy treated with leuprolide acetate

Figure 4. Leuprolide acetate rescues the total number of spines on dendritic outgrowths after ovariectomy

OVX-associated decreases in spine density are rescued by leuprolide acetate treatment in the no delay cohort, and leuprolide acetate increases spine density when a 4 month delay is present between treatment and OVX (4M Delay). A: Leuprolide acetate exhibited an ability to rescue the total number of spines regardless of treatment onset after OVX (one-way ANOVA). B: Representative images of dendritic segments. C: The number of thin spines was rescued when treatment with leuprolide acetate began on the OVX day. D: The number of mushroom spines was increased by leuprolide acetate treatment with a 4 month delay between OVX and treatment onset. Results are expressed as mean $\pm$ sem. Significant difference ($p < 0.05$) from SHAM+SAL (a), from OVX+SAL (b), from OVX+LA (c) and from OVX+E2 (d) SHAM+SAL, sham ovariectomy treated with saline; OVX+SAL, ovariectomy treated with saline; OVX+E2, ovariectomy treated with 17β -estradiol; OVX+LA, ovariectomy treated with leuprolide acetate

Table 1
Leuprolide acetate decreases serum LH levels after OVX

Serum LH levels were analyzed by the UVA Center for Research in Reproduction Ligand Assay and Analysis Core. In both the no delay cohort and the 4 month treatment delay (4M Delay) cohort an increase in serum LH levels was observed with OVX. Treatment with E2 or leuprolide acetate (LA) lowered serum LH levels.

Results are expressed as mean $\pm$ sem.

Serum LH (ng/mL)	No Delay	4M Delay
SHAM+SAL	0.31 $\pm$ 0.12	0.52 $\pm$ 0.15
OVX+SAL	3.29 $\pm$ 0.44	4.01 $\pm$ 0.47
OVX+E2	0.06 $\pm$ 0.02	0.04 $\pm$ 0.00
OVX+LA	0.10 $\pm$ 0.03	0.07 $\pm$ 0.01