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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 17β-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.

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 17β-estradiol (E2) on neuronal structure and function (Woolley and McEwen, 1992) and dysfunction after ovariectomy (OVX) at neuronal (Woolley and McEwen, 1992; Waters et al., 2009; Tanapat et al., 1999) and functional levels (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., 2013), 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

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the classic GnRHR antagonists such as antide in male and female rats (Ziegler and Thornton, 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 postpartus 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.

Methods

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 6 AM. 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 were done in accordance with the Institutional Animal Care and Use Committee of Case Western Reserve University.

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 (Fig. 1).

Ovariectomy and pump implant

Mice were deeply anesthetized and ovaries were either removed or exposed and reinserted (SHAM) bilaterally though a single incision. The animals were subcutaneously implanted with either physiological saline, E2 (1.1 ng 17β-estradiol/day) or leuprolide acetate (3.6 μg 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 66 pg/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 (12-85 pg/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.

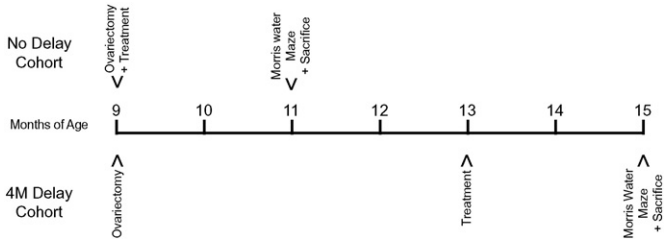

Fig. 1. Schematic diagram for OVX, treatments and Morris water maze. The no delay cohort was sham operated or ovariectomized at 9 months of age and immediately treated with saline, E2 or leuprolide acetate. After 8 weeks of treatment the no delay cohort was trained on the Morris water maze behavior paradigm. 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.

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 10.5 cm diameter platform was covered by 0.5 cm 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 s elapsed, in which case they were gently guided to the platform and given 15 s 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 s 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.

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 h, 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.

Spine density

Hemispheres stored in 30%glycerol 30%sucrose 0.2%NaN_3 were thawed, cut coronally at 250 μm on a vibratome, and placed in PBS in a 12-well plate. A Helios gene gun was used with 1.6 μm 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 Neuronstudio (CNIC, Mount Sinai School of Medicine) and were used to trace apical dendrites, then perform Scholl analysis to classify and quantify spines