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Luteinizing hormone modulates cognition and amyloid-β deposition in Alzheimer APP transgenic mice

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Abstract

Until recently, the study of hormonal influences in Alzheimer disease was limited to the role of sex steroids. Despite numerous epidemiological studies supporting a protective role for estrogen in Alzheimer disease, recent studies show that estrogen administration in elderly women increases the risk of disease. Reconciling these contradictory reports, we previously hypothesized that other hormones of the hypothalamic–pituitary–gonadal axis, such as luteinizing hormone, may be involved in the onset and development of the disease. In this regard, luteinizing hormone is elevated in Alzheimer disease and is known to modulate amyloidogenic processing of amyloid-β protein precursor. Therefore, in this study, to evaluate the therapeutic potential of luteinizing hormone ablation, we administered a gonadotropin-releasing hormone analogue, leuprolide acetate, to an aged transgenic mouse model of Alzheimer disease (Tg 2576) and measured cognitive Y-maze performance and amyloid-β deposition after 3 months of treatment. Our data indicate that luteinizing hormone ablation significantly attenuated cognitive decline and decreased amyloid-β deposition as compared to placebo-treated animals. Importantly, leuprolide acetate-mediated reduction of amyloid-β correlated with improved cognition. Since both cognitive loss and amyloid-β deposition are features of Alzheimer disease, leuprolide acetate treatment may prove to be a useful therapeutic strategy for this disease.
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1. Introduction

Various lines of evidence suggest the involvement of menopause and age-related testosterone decline-induced changes in hypothalamic–pituitary–gonadal (HPG) axis hormone levels in the etiology of Alzheimer disease (AD) (reviewed in [1]). Declines in sex steroids have long been associated with AD incidence and prevalence [2] and hormone replacement therapy (HRT) linked to a decreased risk of developing AD [3,4]. However, recent findings, reporting negative cognitive effects following HRT in women at an AD-vulnerable age [5–7]. Alternative theories also involve the role of free testosterone and high sex hormone-binding globulin (SHBG) levels in the disease such that reduced testosterone levels, as those found in women, and increased levels of SHBG, as those found in HRT takers, which result in reduced free testosterone, may account for the higher incidence of disease in women [8–10]. Nevertheless, changes in estrogen and testosterone do not account for why men with Down's syndrome have a significantly higher risk for developing AD-type changes than women since the levels of sex steroids in individuals with Down's syndrome are comparable to those found in the general population [11,12]. This phenomenon indicates that hormones other than estrogen or testosterone per se may be important. Interestingly, in Down's syndrome individuals have higher levels of gonadotropins such as LH [13], and more importantly, men have higher levels of luteinizing hormone (LH) than do

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age-matched women [11], whereas the opposite is found in the general population (i.e., higher levels of LH in women than in men of the same age [14]). Therefore, LH represents the only factor, thus far, that explains the gender predisposition in the incidence of AD as well as its reversal in Down's syndrome [15]. Supporting this view, individuals with AD have a two-fold elevation in LH serum concentrations when compared to age-matched controls [14,16]. However, two recent studies reported no elevation of LH in AD [17,18]. This disparity in results likely reflects the different ages and grouping techniques used across the studies. This aspect aside, it is important to note that gonadotropin (LH)-based etiopathogenic etiology of AD is supported by several other pieces of evidence. First, questions regarding a "critical period" of HRT-based protection against dementia [19,20] suggest that members of the HPG axis other than sex steroids could be involved. In this regard, the levels of LH are highest during the peri-menopause and early menopausal periods [21] and it is notable that HRT during this period, which by increasing estrogen would lower LH levels, has been observed to be most successful at preventing dementia. Secondly, in the brain, likely due to the fact that LH can cross the blood brain barrier freely [22,23], patients with AD show increased levels of LH in topographical locations that mirror the selective and vulnerable neuronal populations [24]. This latter finding likely relates to the fact that the highest density of LH receptors in the brain are found within the hippocampus [22], a region that is particularly vulnerable in AD, and the degeneration of which leads to early memory loss in the disease ([25,26] for review). In early studies, suggestive of a key role of LH in AD pathogenesis, we showed that LH drives amyloid-β protein precursor (AβPP) processing towards the amyloidogenic pathway in vitro [27]. These findings indicate that gondadotropins such as LH may play an important role in the onset and progression of AD. Therefore, the aim of this study was to examine the capacity of a gonadotropin-releasing hormone agonist, leuprolide acetate, which abolishes the release of LH [27,28], to modulate cognitive performance and amyloid-β deposition in the brains of aged Tg2576 mice carrying the Swedish APP mutation. Aged females as opposed to younger females were used to attempt to mimic the capacity of this drug to modulate advanced stages of AD.

2. Materials and methods

2.1. Animals

We used female 21 month-old non-cycling transgenic mice Tg2576 that over-express the 695-amino-acid isoform of human AβPP containing a Lys670→Asn, Met671→Leu mutation found in a Swedish family with early onset AD. In these animals, levels of amyloid-β begin to rise in the brain by 6 months of age and by 9–10 months they develop senile plaque-like deposits of amyloid-β [29]. All animals were genotyped using a standard PCR protocol [29]. All animals were group housed (n=3/cage), provided ad libitum access to food and water, and maintained on a 12 h light/dark cycle. The experimental groups were chosen in a random fashion and received leuprolide acetate [7.5 mg/kg, slow release (depot) formulation] (n=8) or physiological 0.9% saline (n=5) injected intra-muscularly (IM) twice monthly for a duration of 3 months and all measurements (behavioral/immunocytochemical) were carried out by the observer blind to the treatment. All animals were weighed 3 times during the study and showed no significant weight loss across groups or time. All animals were in good health with no obvious signs of disease, however, four animals died during the course of the study. Weight and death data are summarized in Table 1. We have previously shown that this dose regimen successfully abolishes LH levels in these mice [27].

2.2. LH measurements

Additional animals (n=4/group) of the same age, genotype, gender, and handling to animals tested for behavior were used to determine treatment efficacy of leuprolide acetate on LH serum levels. Blood samples were taken upon sacrifice, centrifuged to collect serum, and LH levels were measured by RIA analysis (Dr. Nett-ARBL-Endocrine Laboratory, Colorado State University).

2.3. Y-maze procedure

We used the Y-maze task (32 cm×10 cm×26 cm) to measure spontaneous alternation behavior, a cognitive parameter [30] previously used by others to measure effectiveness of potential treatments in animal models of AD [31–33] as well as exploratory activity. Each animal was tested prior to treatment (age 21 months) and at the end (age 24 months). As previously described [31], animals were placed in one of three arms of the maze chosen at random across trials, and left to explore all arms of the maze for 5 min during which time the sequence and number of arm choices were recorded. Spontaneous alterations were expressed as a percentage and referred to as the proportion of arm choices that differed from previous choices [34].

2.4. Amyloid-β immunohistochemistry and quantification

For amyloid-β immunohistochemistry, animals were sacrificed with a lethal dose of pentobarbital and their brains removed and fixed in 4% paraformaldehyde. All brains were simultaneously sagitally sectioned (50 μm) across the hippocampus to ensure accurate region sectioning and quantification [35]. Sections were stained free-floating; after a H_2O_2 treatment and blocking serum, sections were immunostained with a primary amyloid-β antibody (4G8, which recognizes the sequence of amyloid-β in the 17–24 region) (0.164 mg/ml) (1:5000 mouse monoclonal) for 24 h (at 4 °C), a goat anti-mouse secondary antibody for 30 min at RT, and avidin–biotin–HRP complex (Vectastain Elite ABC kit, Vector, Burlingame, CA) for 1 h RT. Sections were developed with diaminobenzidine tetrahydrachloride (DAB) with H_2O_2 and mounted on glass slides.

Quantification of amyloid-β deposition was carried out using a Zeiss Axiocam (Munchen-Hallbergmoss, Germany) and compatible image analysis software, KS300 (Carl Zeiss Vision GmbH, Munchen-Hallbergmoss, Germany). For each animal, every 6th section of a series through the dorsal hippocampus (approximately 240 μm apart) was selected (approximately 11 sections/animal) and quantified for amyloid-β deposition as previously described [36]. Briefly, using a 5× objective, a single field encompassing the entire hippocampus was manually selected and positive staining was expressed as the percent area stained across the area. The values obtained from all sections per animal were averaged.

2.5. Statistical analysis

Using appropriate software (Sigmastat, SPSS-Inc., Chicago, IL), a two-way repeated measures ANOVA was used to determine differences in Y-maze alternation behavior across treatment groups (leuprolide acetate vs. saline) and

Table 1
Weight and animal death information

	Number of animals		Weight (g)	
	Baseline	3 months	Baseline	3 months
Saline	8	5	32.1±3.7	28.8±3.3
Leuprolide	9	8	27.8±1.0	28.7±1.7

time (Baseline and 3 months) as a repeated measures factor. The Student's *t* test was used to determine differences in amyloid-β burden between leuprolide acetate and saline-treated animals.

3. Results

Leuprolide acetate treatment, in accord with previous data [27,28], significantly lowered LH secretion in our model ($P<0.02$) (Fig. 1). Importantly, these declines led to sustained cognitive ability as measured by spontaneous alternation behavior in the Y-maze task when compared to saline treatment ($F_{1,11}=14.745$, $P<0.01$) (Fig. 2). Specifically, while both groups were comparable at baseline ($t_{1,11}=0.944$, $P<0.35$), leuprolide acetate-treated animals showed significantly lower rate of decline when compared to saline treated animals at 3 months post-treatment ($t_{1,11}=29.438$, $P<0.02$). Importantly, sustained performance was present in the absence of differences in locomotor activity across groups (total number of arms entered/5 min trial) ($F_{1,11}=0.670$, $P<0.430$).

A Student's *t* test statistical analysis used to determine differences in amyloid-β deposition in treated and non-treated groups revealed that leuprolide acetate treatment significantly decreased the levels of amyloid-β deposition in the hippocampi of treated versus non-treated aged animals ($t_{1,10}=-3.782$, $P<0.01$) (Fig. 3). Notably, reductions in amyloid-β were significantly correlated (Pearson's analysis) with improvements in cognitive performance ($r=-0.75$, $P<0.05$), such that leuprolide acetate-mediated decreases in levels of amyloid-β deposition were associated with improved performance in the Y-maze task.

4. Discussion

Our data reveal that treatments that target HPG axis hormones such as LH can modulate cognitive behavior in aged AβPP transgenic mice, and also decrease the extensive deposition of amyloid-β. These results support our hypothesis that HPG axis function, and in particular the changes that occur later in life (i.e., elevations in LH following menopause/age-related declines in testosterone), may play an important role in the pathogenesis of AD [1,15,37,38].

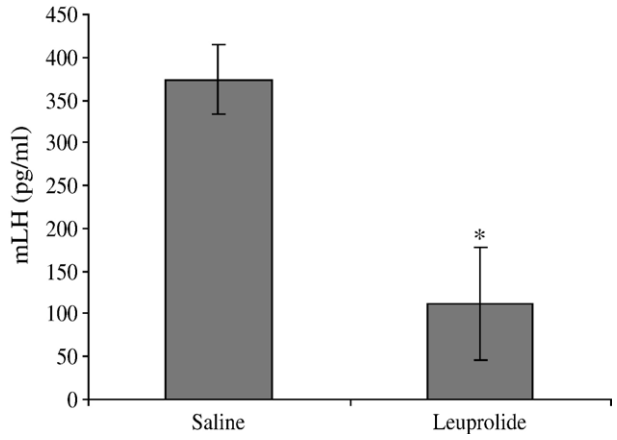

Fig. 1. Serum mouse LH levels (mLH pg/ml ± SEM) measured by RIA for saline and leuprolide acetate-treated animals ($n=4$/group). *Indicates significance at $P<0.02$.

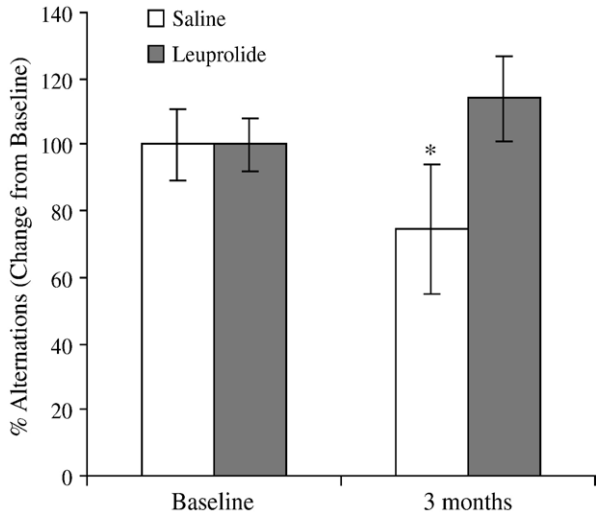

Fig. 2. Y-Maze performance in Tg2576 mice after leuprolide acetate ($n=8$) or saline treatment ($n=5$) at baseline and after 3 months. Figure illustrates the mean % alternations expressed as % change from baseline. * Indicates significance at $P<0.05$.

Coupled with the decreases in LH, leuprolide acetate treatment leads to decreases in sex steroids such as estrogen which have been associated with declines in cognitive output [39–44]. Therefore, our data suggest that, at least in aged AβPP transgenic mice, the positive effects of LH ablation override any negative effects of estrogen depletion. Indeed, as shown in this study, leuprolide acetate treatment maintains alternation behavior in the Y-maze task, which has been interpreted to reflect intact working memory. Alternation behavior also depends on the animal's innate tendency/preference to alternate, leading to the possibility that treatment, rather than improving/sustaining memory, could increase alternating preference. The fact that our data show sustained rather than improved behavioral output in the treated animals compared to controls and the fact that treated animals did not show increases in overall arm entries nor any directional biases, suggests that treatment did indeed sustain short-term memory rather than potentiate their preference to alternate. Such an assertion is in concert with data demonstrating that the modulation estrogen in the AβPP/PS-1 animal model of AD leads to improvements in cognitive behavior but, and unlike our findings, no changes in pathological features of AD [45]. This slight discrepancy in results can be explained by a differential LH status in the animals of the two studies since while in our study we ablated both estrogen and LH concurrently, ovariectomy [45] leads to declines in estrogen but a rise in the levels of LH and administration of estrogen (c.f. HRT) does not decrease LH levels beyond baseline. Therefore, one possibility is that it is only the decrease in estrogen when it is coupled with an increase in LH that leads to behavioral impairments and it is only the ablation of LH that leads to changes in amyloid-β pathology in these mice. Additionally, such differences could

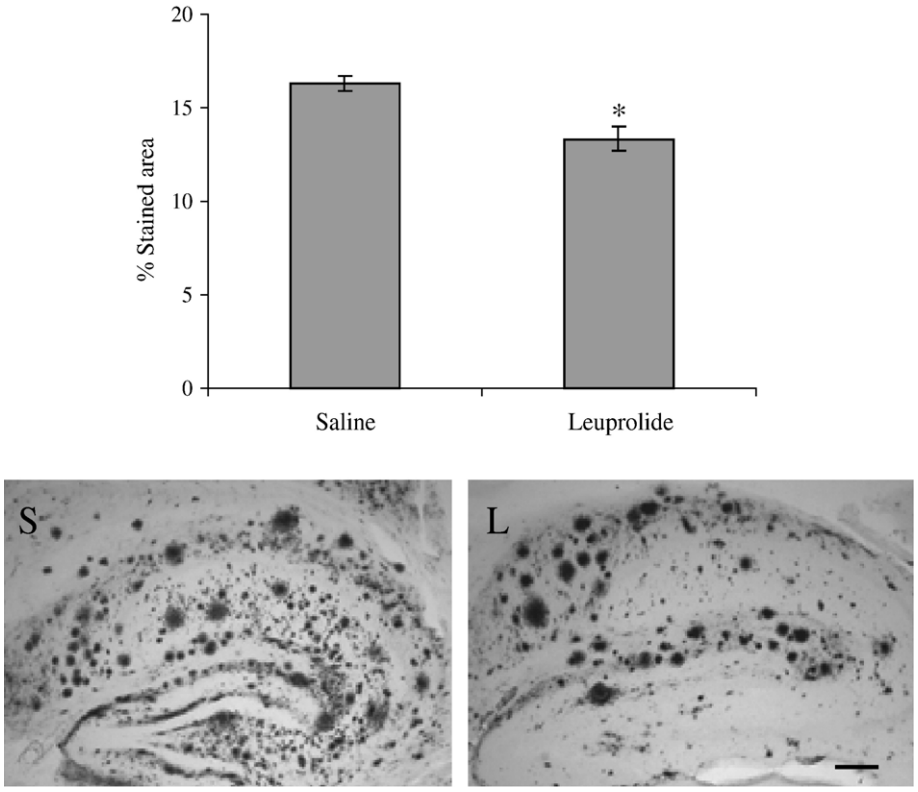

Fig. 3. Amyloid-β burden measured as % area stained in the entire hippocampus of 11 sections/brain/animal is significantly lower in animals treated with leuprolide ($n=8$) compared to saline-treated animals ($n=5$, $^*P<0.05$). Representative image of amyloid-β-burden in Tg2576 mice after saline (S) or leuprolide (L). Scale bar, 200 μm.

also arise from the differences in techniques used (silver stain vs. 4G8, a non-human specific antibody). Clearly, future, more in depth, studies targeting all of these variables should be carried out to clarify the interactive role of these hormones on cognition and AD-related pathology. That withstanding, the data do provide compelling evidence that LH is an important component in the pathophysiological processes of AD.

The precise mechanistic pathway by which LH is involved in AD is still under investigation, nonetheless, that LH modulates the processing of AβPP [27] may be important given the proposed central role of amyloid-β in AD pathogenesis [46] or its proposed indirect role as a surrogate marker of neuronal health [47,48]. In this regard, the reduction of amyloid-β and coincident sustainment of alternation behavior following LH ablation, observed in this study, is in agreement with previous studies showing a correlation between amyloid-β burden and cognition [49,50]. Importantly, given the high density of LH receptors in the hippocampal region [23] and the higher levels of LH in the brains of patients with AD [14,16] it is likely that LH plays a significant role in the pathogenesis of the disease dependent, as well as independent, of amyloid-β-related processes.

Based on the aforementioned notion that LH is a driving pathogenic force in AD, leuprolide acetate, a gonadotropin-releasing hormone agonist, which suppresses LH to undetectable levels by down-regulating pituitary gonadotropin-releasing hormone receptors [27,28], might be an effective method of treatment for patients with AD. A previous study in humans demonstrated that treatment with leuprolide acetate led to initial increases in serum concentrations of amyloid-β 1–40 [51]; this led to the assumption that declines in sex steroids by chemical castration could lead to an increase in AD pathogenesis. However, it is important to note that leuprolide acetate is a gonadotropin-releasing hormone agonist which initially increases the levels of LH before the levels decline and therefore, an initial increase in LH could account for the initial increases in serum amyloid-β observed. On the other hand, increased serum amyloid-β may also reflect increased efflux of amyloid-β from brain, as has been documented in transgenic AβPP mice after immunotherapy [52].

Importantly, to reflect a realistic therapeutic window as it would apply to human patients, in our study we used aged mice where cognitive decline and amyloid deposition are both evident. In addition, and without attempting direct comparisons across species, studying aged rather than young female mice provided a similar hormonal environment to that of a post-menopausal female (i.e., deregulation and responsiveness to gonadotropins as well as low estrogen levels), which is typically the population that develops AD [53]. However, in order to study the full spectrum of leuprolide acetate treatment effects, future studies should include a young group as well as an ovariectomized group. Certainly, the complex interaction between all the HPG axis components suggests that it is unlikely that any one hormone of this axis plays a single and predominant role but rather it may be the balance or ratio of one hormone to another, i.e., LH to estrogen or testosterone. In this

regard, individuals with higher LH to sex steroid ratios such as women [54] and men [55] with lower endogenous sex steroid levels (i.e., high LH to estrogen ratio) show higher rates of AD and would likely benefit from leuprolide acetate treatment more than women/men with higher endogenous levels of estrogen/testosterone (lower LH:estrogen ratio), who have lower rates of AD. Likewise, in men, treatment of leuprolide acetate could be beneficial albeit masked by the effects of testosterone depletion [56], in which case, testosterone replacement could be ideal. Therefore, and considering that the totality of hormonal influences cannot be ignored, our study demonstrates that targeting LH with leuprolide acetate, a product already safety-approved for therapeutic use in prostate cancer, is as effective if not more effective, as shown by its capacity to both positively modulate cognition and reduce amyloid-β load, than estrogen therapy alone. Likewise, the fact that leuprolide acetate was capable of modulating cognitive behavior and reducing advanced amyloid-β deposition in aged animals suggests that this treatment may be an effective treatment strategy even at late stages of disease. In this regard, a recently completed phase II clinical trial (http://clinicaltrials.gov/ct/show/NCT00076440?order=6) indicates that patients treated with high doses of leuprolide acetate show a stabilization in cognitive decline (ADAS-Cog, ADCS-CGIC) and activities of daily living (ADCS-ADL) (http://www.secinfo.com/d14D5a.z6483.htm, pp. 56–64), therefore our findings are in agreement with those in human trials.

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