Prolame ameliorates anxiety and spatial learning and memory impairment induced by ovariectomy in rats

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A B S T R A C T

N-(3-hydroxy-1, 3, 5 (10) estratrien-17beta-yl)-3-hydroxypropylamine (17β aminoestrogen, prolame) is a steroidal compound with weak estrogen-related trophic-proliferative effects in uterus. Contrasting with 17β-estradiol (E2) pro-coagulant effects, this compound has high anticoagulant and antiplatelet effects. It has been extensively demonstrated that E2 plays important roles in brain function. However, prolame’s influence on central nervous system has not been documented. In this study, we evaluated the effects of prolame replacement in young ovariectomized rats on spatial learning and memory and anxiety, correlating pyramidal cell dendritic spine density changes and neuronal nitric oxide synthase (nNOS) expression in the hippocampus. Ovariectomized young rats were treated with prolame for 4 weeks. Three other groups were used as physiological, pathological, and pharmacological references as follow: gonadally intact cycling females, ovariectomized, and ovariectomized with 17β-estradiol treatment respectively, for the same time period. Experiment 1 investigated the behavioral effects of prolame on anxiety and spatial learning using elevated plus maze (EPM) and Morris water maze (MWM) paradigms respectively. Experiment 2 studied the dendritic spine density and neuronal nitric oxide synthase expression in the hippocampus of the 4 experimental groups. Similar to estradiol, prolame reversed the anxiogenic effects of ovariectomy, evaluated by EPM, and enhanced MWM performance to the level of gonadally intact subjects. Hippocampi from prolame-treated rats exhibited enhanced nNOS immunoreactivity and its relocation in dendritic compartments, as well as recovery of dendritic spine density loss in pyramidal neurons. Hence, prolame may provide an alternative option for ameliorating neurological symptoms caused by surgical menopause.

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1. Introduction

Estrogens regulate a host of physiological processes, but most important among those that affect reproduction and basic physiological functions are mammary gland development, reproductive success, hypothalamus–hypophyseal functions, bone integrity, cardiovascular protection, and immune response [1]. Two forms of the nuclear estrogen receptor (ER) are found in mammalian species. These two receptor subtypes are products of different genes and are designated ERα (ERα) and ERβ (ERβ). Both monomeric forms of each receptor subtype are approximately 60 kDa, and both subtypes bind 17β-estradiol (E2) with high affinity, but differ in DNA-binding affinity [2]. The ERα and ERβ can be co-expressed in some target tissues, but they also exhibit different tissue or cell expression patterns. A small amount (around 3%) of ERs are found to be located in the plasma membrane [3] and treatment with E2 stimulates a phosphoinositide cascade as well as activation of adenylate cyclase, suggesting that the ERs are coupled to Gαq and Gαi [3,4].

On the other hand, while estrogen’s effects on mammal’s peripheral physiology have been extensively explored and characterized, our knowledge on estrogen’s influence on central nervous system (CNS) and its consequences on cognition and behavior are still fragmentary and are currently being intensively studied. E2 exerts potent and wide-ranging effects on the morphology and function in different regions of the CNS [5]. Brain targets for E2 effects on anxiety and depression include the hippocampus and amygdala. Administration of E2, compared to vehicle, subcutaneously or directly into the hippocampus or amygdala of ovariectomized rats decreases anxiety and depressive behaviors [6,7].

Estrogen deficiency due to natural or surgical menopause is associated with cognitive and emotional impairment [8]. Hormone replacement therapy (HRT) has proven to be effective in preventing and reversing

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some of these conditions, including neurological impairments such as spatial memory and learning deficiencies [9], development of anxiety-like behavior [7], and (at a microscopical level) the loss of synaptic and dendritic spine density [10]. However, the increased risk of breast cancer [11] and venous thromboembolism [12,13] produced by conventional estrogenic treatment remains a major drawback and a strong motive to find new therapeutic strategies. This need has been underscored in the Women’s Health Initiative (WHI) [14].

The monoamino-estrogen N-(3-hydroxy-1, 3, 5 (10) estratrien-17beta-y1)-3-hydroxypropylamine (prolame) belongs to the group of 17β-aminoestrogens-estradiol analogs, such as prodiame, buame, and pentolame [15]. These estrogen derivatives possess a modification of the steroid nucleus in the C-17-amino-side chain position (with an increased length in the amino-alcohol side chain). Since it was initially synthesized in 1985 [16] this steroidal compound has been shown to have low estrogen-related trophic-proliferative effects in uterus [17] [18] and prolonged anticoagulant/antiplatelet effects [17,19,20]. These results seem to be linked to an increased nitric oxide (NO) production by platelets and endothelial cells [21]. It has been reported that this amino-estrogen compound activates ER-dependent reporter gene expression, preferentially mediated through the ERα, whereas less activation occurred through the ERβ [17]. However, the effects of this amino-estrogen on the CNS have not been properly evaluated, except for a recent paper where Lemini and Canchola [22] reported that prolame promotes sexual behavior in ovariectomized young female rats. Ovariectomy, surgical removal of the ovaries, is a common animal model for studying postmenopausal changes in adult female rats due to ovarian hormone loss [7,23]. This procedure has been utilized mainly to assess E2 behavioral effects. Ovariectomy increases anxiety and depressive-like behaviors and impairs spatial learning and memory. Subcutaneous administration of E2 can reverse these effects in several tasks [8,24–27].

It has been shown that NO mediates neuronal signaling and processes involving synaptic plasticity, such as long-term potentiation (LTP), widely considered one of the major cellular mechanisms underlying learning and memory [28–30]. NO is able to facilitate synaptic transmission in the hippocampus, contributing to LTP [31,32]. NO formation, by neuronal NO synthase (nNOS), has been shown to depend on the estrogen mediated association of nNOS with NMDA receptors, which enhances nNOS activation in preoptic neurons [33]. Estrogen increases spine density via activation of NMDA receptors [6], and triggers nNOS expression (for a review see [34]), inducing the activation of NO signaling, which may contribute to learning and memory.

In this study, we hypothesized that chronic prolame administration enhances the production of nNOS, which reverses the detrimental effects on dendritic spine density produced by chronic loss of estrogen levels in the brain, diminishing anxiety and spatial learning and memory deficiency caused by ovariectomy. Our control/reference groups included: gonadally intact cycling female rats, ovariectomized rats without any treatment, and ovariectomized rats with E2 treatment.

2. Materials and methods

2.1. Prolame synthesis

The detailed process for prolame synthesis was described elsewhere [35]. Briefly, a toluene solution of 3-hydroxypropylamine and estrone was refluxed for 12 h with a Dean–Stark trap and then vacuum concentrated. The obtained solid product was dissolved in methanol, and reacted with sodium borohydride. The obtained product was filtered, washed with water and recrystallized from methanol–water. Estrone, 3-hydroxypropylamine, toluene, methanol and sodium borohydride were purchased from Sigma-Aldrich Chemical Co (St. Louis MO, USA). The structural formula of prolame is depicted in Fig. 1.

2.2. Animals

Wistar rats from the Faculty of Medicine, UNAM animal facility were used in this study. All animal procedures were approved by the local ethical committee (Comisión de Investigación y Ética de la Facultad de Medicina, UNAM, ID 044-2011), in accordance with the principles exposed in the Handbook for the Use of Animals in Neuroscience Research (Society for Neuroscience. Washington, D.D.1991). Animals were housed in an artificial 12-h light schedule (light on at 20:00) in a room with temperature between 20°C and 24°C with adequate ventilation and given access to standard rat chow and water ad libitum.

Forty-eight female rats were used in this study. Animals were assigned to the following groups: gonadally intact cycling (GI, n = 12), ovariectomized (Ovx, n = 12), ovariectomized plus estradiol treatment (OvxE, n = 12) and ovariectomized plus prolame treatment (OvxP, n = 12). The GI, Ovx, and OvxE groups served as the physiological, pathological and pharmacological reference groups. Rats were bilaterally ovariectomized according to procedures described elsewhere [36], except for GI group, which only received sham surgery. These procedures were performed at postnatal day 90 (PN90) under i.p. ketamine (90 mg/kg) and xylazine (10 mg/kg) anesthesia.

2.3. Prolame and E2 treatment

Daily subcutaneous injections of E2 (Sigma-Aldrich Co, E8875, 50μg/kg) and a molar equivalent dose of prolame (60 μg/kg) were applied for a month to the OvxE and OvxP groups respectively, starting 4 weeks after the surgery. The dose of E2 was determined according to previous studies [37]. Treatments were applied for a month to the OvxE and OvxP groups respectively, starting 4 weeks after the surgery. The drugs were diluted (10%) in vehicle containing glycerol and distilled water 1:1. GI and Ovx groups received vehicle only. It is worth mentioning that we used a model of 4-week delay between Ovx and hormone replacement, which was determined for the purposes of this study. This delay is within the 2 to 8 week window in which acute estrogen replacement has previously shown to influence behavior effectively [38].

2.4. Experiment 1—effects of prolame treatment on anxiety-like behavior and spatial memory performance

The behavioral tests were conducted after 1 month of treatment, during animal activity period (light-off period).

2.4.1. Elevated plus maze test (EPM)

EPM was used to assess the unconditioned acute anxious state and the procedure was described elsewhere [39]. Briefly, the plus maze was made from wood with conventional dimensions. The only relevant modification is that the open arms were surrounded by an upwards-protruding edge of 0.5 cm. This latter measure prevents the rat from falling accidentally without jeopardizing the elemental features of the setting. The EPM was lit with dim red light and monitored by closed-circuit television (CCTV). The maze was cleaned with neutral detergent and water and dried before each rat was tested.
Prior to the test, rats of each experimental group were habituated by handling and placing them in an open field box (50 cm × 50 cm × 50 cm, made from wood) for the previous three days and immediately before the EPM, 5 min per session. This procedure is based on previous observations from our group that gentle handling and pre-exposure to a novel environment before testing in the EPM increases the motor activity and the likelihood of entering to the open arms of the maze [39]. This procedure was also reported previously in the literature [40]. At the beginning of the test, rats were placed on the center of the maze heading to an open arm and then left for free exploratory activity during 5 min. The total time spent on the open arms (with all limbs placed out of the central square) was the operational definition of unconditioned anxiety (exploration vs. avoidance). The percent of the 5-min test time spent in the open arms was the dependent variable used for statistical analysis.

2.4.2. Morris water maze test (MWM)

The MWM [41] was conducted the day after the EPM. A black circular pool (156 cm diameter, 80 cm high) filled with 30 cm height of water (25 °C ± 1 °C) was used for this cognitive test. Some visual cues were placed on the pool wall. A circular black escape platform (12 cm diameter) was submerged 1 cm below the water surface. Rats were habituated to the MWM by 5 training trials performed one day before the test. These training trials followed the same protocol mentioned below, except for a different location of the platform. On the day of the test, rats were placed on the center of the maze heading to an open arm and then left for free exploratory activity during 5 min. The total time spent on the open arms (with all limbs placed out of the central square) was the operational definition of unconditioned anxiety (exploration vs. avoidance). The percent of the 5-min test time spent in the open arms was the dependent variable used for statistical analysis.

For cell counting, eight slices from each rat were taken at 150 μm interval. Two independent researchers who were blind to the experimental conditions carried out cell counting. Principal neurons located in CA1, CA3 and dentate gyrus (DG), immunoreactive to nNOS were counted using a modified method from [48,49]. Nikon ECLIPSE 50i fluorescence microscope at 40× objective with B-2A longpass emission filter was used. Counting of nNOS+ cells was performed under microscope, inside a length of 540 μm (corresponded to the diameter of the 40× objective field) of the cell body layer, i.e. pyramidal layer for CA1 and CA3, and granule cell layer for DG. Neurons were differentiated from glial cells by the morphological characteristics of pyramidal and granule cells. Due to the cellular heterogeneity of the hippocampal subregions, counting fields were not chosen randomly but 2 fields per each subregion (i.e. CA1, CA2/3 and DG) per section, namely, from the CA1-CA2 border, two consecutive fields to CA1 direction for “CA1” counting and two consecutive fields to CA2/3 direction for “CA2/3” counting. For DG granule cell layer only the supra-pyramidal segment in the hilar region was chosen for counting. This criterion was set to minimize the possible errors due to the anatomo-functional heterogeneity of studied region.

The remaining halves of the rat brain tissue underwent Golgi–Cox stain protocol to assess dendritic spine densities of hippocampus projection neurons. The detailed procedure was described elsewhere [50]. Briefly, the central one-third parts of the forebrain, along antero-posterior axis, were cut to form blocks of approximately 10 mm between the rostral and caudal edges. Tissues were rinsed briefly in PB and then immersed in sequenced impregnation solutions following the fabricant’s indications (FD Rapid GolgiStain kit, FD NeuroTechnologies, Ellicott City, MD, USA) for 2 weeks in the dark. Sections of 150 μm were sliced using a vibratome and mounted on gelatine-coated glass slides. These sections were dried naturally at room temperature in the dark for at least two days and then stained with a staining solution included in the kit mentioned above. Samples were followed by dehydration and processed for permanent mounting with Permount (Electron Microscopy Sciences, PA, USA) mounting medium.

Spine densities were counted on the primary basilar branches, classified as left/right basilar and primary, secondary and tertiary apical dendritic branches from CA1 and CA2/3 pyramidal neurons at 100x light microscope and traced with the help of a drawing tube. Six randomly chosen segments of 10 μm length per each subject/hippocampal subregion/dendritic branches were analyzed, n = 30, i.e., 5 subjects × 6 segments in each classified dendritic branch.

2.6. Statistical analysis

Quantitative results were expressed as mean ± standard error of mean (SEM). Multiple pair-comparisons were performed using Tukey’s test after ordinary one-way analysis of variance (ANOVA), except for the MWM data where repeated measures ANOVA was performed, using ‘trials’ as the within-subject factor and ‘treatment’ as between-subject factor, followed by Tukey’s test. Post-hoc differences were considered statistically significant at a value p < 0.05 (*p<0.05, **p<0.01, ***p<0.001). The analysis was done using SPSS release 9.0 software.
3. Results

3.1. Experiment 1. Prolame reversed anxiety-like behavior exhibited during EPM

After 1 month of treatment, EPM was used to assess anxiety-like behavior. Fig. 2 depicts the EPM performance of the 4 tested groups, expressed as percentage of time spent in the open arms during the 5-min test. A one-way ANOVA showed that the time spent on the open arms differed significantly between the treatment groups, F(3, 44) = 5.174, p = 0.0036. Furthermore, post-hoc Tukey’s multiple comparison test showed that gonadally intact cycling (GI) (29.13 ± 3.45) and prolame-treated (OvxP) rats (33.91 ± 2.87) spent significantly more time in the open arms than ovariectomized (Ovx) rats (16.64 ± 2.86). All other comparisons were not significant.

3.2. Prolame treated subjects exhibited enhanced spatial learning and memory during MWM

Following the EPM, the rats underwent MWM habituation test as described in Materials and methods. All groups acquired the platform location, as supported by decreased latency to locate the platform (or decreased swimming time to the platform) across the 8 trials (Fig. 3). There was no apparent difference regarding swimming speed. Repeated measurements analysis showed statistical differences among trials (multivariate tests F(7, 38) = 27.75, p < 0.0001), among treatments (F(3, 44) = 4.95, p = 0.005), but no interaction between trial and treatment (multivariate tests F(7, 40) = 1.651, p = 0.485). Post-hoc Tukey’s analysis showed that trials from 3 to 8 were statistically different (p < 0.05) from the first trial, in this trials GI, OvxE and OvxP rats found the platform faster than Ovx rats. (For Ovx vs GI p = 0.017; for Ovx vs OvxE p = 0.051 and for Ovx vs GI p = 0.008).

3.3. Prolame-treated subjects exhibited enhanced nNOS immunoreactivity and dendritic relocation in the hippocampus

Neurons from CA1, CA2/CA3 and the DG cell body layers expressing nNOS were counted in the dorsal hippocampus (Fig. 4A). A one way ANOVA showed a significant effect of treatment on CA1 (F(3,316) = 148.9, p < 0.0001), CA2/CA3 (F(3,316) = 65.96, p < 0.0001) and DG (F(3,316) = 41.89, p < 0.0001). Post-hoc analyses showed a reduction in the number of neurons that express nNOS in Ovx compared to GI in all areas (p < 0.001). However nNOS expression was restored in both OvxE and OvxP (p < 0.001) (Fig. 4A, right panel).

3.4. Prolame-treated subjects exhibited dendritic spine density recovery of pyramidal neurons

A one-way ANOVA showed a significant effect of treatment on dendritic spine density of pyramidal cells in CA1, on the right basilar branch (F(3,116) = 19.38, p < 0.0001), left basilar branch (F(3,116) = 23.53, p < 0.0001), secondary apical branch (F(3,116) = 48.89, p < 0.0001) and tertiary apical branch (F(3,116) = 62.90, p < 0.0001).

CA2/3 also showed differences on the right basilar branch (F(3,116) = 24.23, p < 0.0001), left basilar branch (F(3,116) = 63.66, p < 0.0001), secondary apical branch (F(3,116) = 17.63, p < 0.0001) and tertiary apical branch (F(3,116) = 239, p < 0.0001).

Post-hoc analyses showed higher dendritic spine density in GI (right and left basilar, secondary and tertiary), and OvxE (right basilar, left basilar, secondary and tertiary) in comparison with Ovx. Prolame reversed dendritic spine loss in OvxP, in particular in the left basilar, secondary and tertiary branches (Fig. 5A) of CA1 and the right basilar, left basilar, secondary and tertiary branches (Fig. 5B) of CA2/3.

4. Discussion

The present study demonstrated for the first time that prolame, which has previously showed to have weak estrogenic effects, 22- to 36-fold less potent than estradiol in uterus [18], can reverse anxiety-genesis and impaired spatial learning and memory produced by ovarian hormone loss in ovariectomized young rats. Furthermore, prolame was able to increase nNOS expression and recover the dendritic location of this enzyme in the hippocampus in a similar way as E2. Moreover, the reduction of dendritic spines in the principal neurons due to the loss of ovarian hormones was also restored after prolame treatment, matching the levels of GI and E2 treated rats.

Estrogens have many effects in the central nervous system, including effects on anxiety and depression behavior. Results of the EPM experiments confirmed previous reports in which the loss of ovarian function caused by surgical removal of ovaries, causes anxiety-like behavior [51].
Furthermore, prolame treatment reversed anxiety-like behavior, matching the effects of estradiol on EPM. Estrogenic signaling on anxiety may depend upon many factors. Administration of E2, compared to vehicle, subcutaneously or directly into the hippocampus or amygdala of ovariectomized rats decreases anxiety and depressive behavior (for a review see [7]). Administration of an ER antagonist to the hippocampus, but not to the amygdala, increases anxiety and depression behavior of naturally receptive female rats [7]. Results from this study support previous findings and suggests that prolame could reverse the anxiogenic effects caused by ovarian hormone loss.

In the MWM, our data confirmed a recent report [52], which showed that ovarian hormone loss due to ovariectomy in rat impairs MWM performance, revealed by escape latency. Results from our study showed that the Ovx group had significant increases in escape latencies at least three trials compared with all three other groups, i.e. GI, OvxE and OvxP. There were no significant differences at any time point among the latter ones, demonstrating that prolame effectively repaired the detrimental effect of ovariectomy on this spatial learning and memory assessment, so as E2 treatment. Interestingly, this recent report [52] also showed that the use of nitric oxide synthase (NOS) inhibitor, N(G)-nitro-L-arginine methyl ester (L-NAME) abolished the estradiol-mediated restoration of spatial learning and memory in ovariectomized rats evaluated in MWM. This result indicated an interaction of NO and E2 in this specific brain function. Our results concerning NOS expression in hippocampus showed a significant loss of nNOS positive cells in the pyramidal and granular cell layers of the Ovx group, in which nNOS expression was exclusively found in the cell bodies. Treatment with both E2 and prolame reversed these detrimental effects, which is consistent with the improvement of MWM performance. These data, together with the results published by Azizi-Malekabadi et al., suggest that the modifications in nNOS expression and MWM performance produced by prolame could have a causal relationship. Concerning the distinct subcellular localization of nNOS, the expression of this enzyme in the Ovx
while silencing neuronal activity favors a nuclear localization [53]. NOSIP was found after NMDA receptor-evoked activity of neurons, localization by interacting with nNOS. A non-nuclear localization of NOS interacting protein (NOSIP) modulates nNOS subcellular
distribution we observed closely resembled that of Dreyer et al. [53], where found in dendritic spines and interacts with several proteins. The distribution observed closely resembled that of Dreyer et al. [53], where NOS interacting protein (NOSIP) modulates nNOS subcellular localization by interacting with nNOS. A non-nuclear localization of NOSIP was found after NMDA receptor-evoked activity of neurons, while silencing neuronal activity favors a nuclear localization [53]. Taken together, these results indicate that prolame and E2 treatments normalize nNOS subcellular localization, probably promoting NMDA receptor-evoked activity of hippocampal neurons, thus regulating NO production.

Analysis of the results obtained by quantification of dendritic spine density showed that prolame and E2 reversed dendritic spine loss in the hippocampus. This may be explained by the fact that estrogens, and probably prolame too, can activate phosphatidylinositol-3-OH kinase (PI3K)/Akt signaling pathways, mainly by nuclear- and membrane-mediated ERs [54–56]. Indeed, Akt activation has been observed in multiple brain areas and has been associated with spine formation. Additionally, Akt pathway plays a well-known role in cell survival, and has also been implicated in estrogen’s neuroprotective actions (for a review see [6]). Akt has a number of downstream targets including the anti-apoptotic member Bcl-2 and endothelial NOS, among others. Recently, the GABA A receptor has been shown to be a substrate of Akt. Phosphorylation of GABA A receptor by Akt increases its cell surface expression, further linking ER-mediated PI3K/Akt signaling with regulation of synaptic plasticity.

Interestingly prolame, which has been demonstrated to have weak estrogenic effects compared to E2 [18], had similar effects on the parameters measured in this report. Indeed, in vitro experiments have shown that prolame is less able to: 1) displace E2 bound to cytosolic ER and 2) activate ER dependent reporter gene CAT expression in comparison with estradiol [17], although it is possible that the degree of inhibition observed may represent an underestimation, due to the in-vitro-experiment limitations. Nevertheless, decreased ER activation results in diminished ER protein expression in the hippocampus [57]. As a result, we would have expected a reduced prolame responsiveness, but this was not the case. One option may be that prolame structure may alter its interaction with ERs, changing its binding to promoter sites and activating transcription of important estrogen responsive genes as efficiently as E2. Another possibility could be that the negative regulation of the genomic and non-genomic mechanisms induced by this amino-
estrogen is reduced in comparison to E2, promoting an enhanced responsiveness. A third possibility could be that prolame effects may be more dependent on non-genomic mechanisms, using multiple membrane-associated ERs or novel C-protein-coupled E2 receptors, such as the GPR30, that responds to estrogen with kinase activation as well as transcriptional responses [58–60]. To elucidate the mechanisms underlying prolame’s influence in CNS further molecular biology studies are required.

5. Conclusion

Prolame treatment of ovariectomized rats has important effects on the central nervous system, as shown in this study. The behavioral effects include restoring spatial learning and memory and ameliorating high anxiety caused by ovariectomy in young rats. At anatomical neuropharmacology level, we showed an enhanced expression of nNOS and restoration of dendritic spine density in the principal neurons of the hippocampal formation.

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