Research Article

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Administration of Anastrozole to Ovariectomized Rats Impairs Working Memory in Association with Plastic Changes to Dendritic Spines on Prefrontal Third-layer Pyramidal Neurons

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Abstract

Gonadal estrogens influence several neurobiological events related to synaptic plasticity underlying cognitive behavior. Likewise, estradiol synthesized in neurons affects aspects of brain organization associated with cognition. Here, plastic changes to dendritic spines on third-layer pyramidal neurons from the prefrontal cortex of ovariectomized, anastrozole-treated female rats were studied. Anastrozole treatment dampened the efficiency of rats in resolving a spatial working memory test in the "Y" maze when compared with ovariectomized control and proestrus female rats. In addition, the administration of Anastrozole led to an increase in dendritic spines and filopodia on the pyramidal cells studied. The thin, mushroom, stubby and wide spines remained unchanged. Since filopodia are considered to be the precursors of novel spines, the increase in dendritic spines is consistent with the increase in filopodia. However, this was clearly insufficient to drive proper working memory performance, despite the apparent stability in the translation of synaptic information suggested by the similar spine types evident in the ovariectomized controls and the increases observed. These findings show that brain-derived estradiol is necessary for prefrontal activity to account for working memory performance. Further studies will be needed to elucidate the mechanisms underlying such spine enhancement in the absence of estradiol-mediated modulation of plasticity.

Keywords: Anastrozole; Estrogen; Dendritic spines; Plasticity; Working memory; Prefrontal cortex

Introduction

Estrogens, mainly 17 β estradiol (E2), have recognized trophic and regulatory roles on the cytoarchitecture, connectivity and function of brain structures. Their activity not only influences events related to sexual behavior and reproductive in adult females but also, neural structures and processes that support other important brain activities like neuroprotection, or learning and memory [1-4]. These effects on neural tissue may be exerted by estrogens synthesized in the ovary, although E2 synthesized in several cerebral structures may also account for some estrogenic actions in the brain [1,5,6]. Thus, both endocrine and paracrine pathways supplying E2 may be involved in its regulatory activity in specific neural structures.

The pathway of steroidogenesis that ends in the synthesis of testosterone is active in neural and glial populations within specific brain structures, driving its metabolic conversion to 5 α -dihydrotestosterone by 5 α -reductase or to estradiol through the activity of aromatase. Thus, it is likely that these "neurosteroids" can modify neural structure and function in these domains [7-12]. Indeed, testosterone aromatase activity has been shown to drive E2 synthesis in both the hippocampus and the prefrontal cortex of male and female rats, even in the absence of gonadal steroidogenesis [13-16]. These brain structures are mainly involved in different modalities of cognition in the brain and therefore, the estrogenic activity exerted by

the E2 produced through ovarian and/or brain steroid synthesis can modify the structural and biochemical neural substrates of cognitive functions [17-20]. Cytoarchitectonic characteristics can be modified by estrogen-related phenomena, including: changes in dendritic spine density and in the proportion of the different spine types on the dendritic branches of pyramidal neurons; synaptic connectivity, activity and strength in hippocampal and prefrontal cortex neuronal circuits. Thus, hippocampal- and prefrontal cortex-dependent cognitive functions are likely to be susceptible to variations in E2 concentration and activity [17,21,22].

Indeed, there is experimental evidence that supports an important role of the estrogen E2 synthesized through testosterone aromatization in these brain structures and its involvement in cognitive functions. Accordingly, inhibition of brain aromatase activity provokes structural and functional alterations to the neural substrate involved in hippocampal- and prefrontal cortex-dependent cognitive functions, leading to deficient performance of both experimental animals and humans in modality-specific learning/memory tests [19,23-30].

In the light of these findings, this study was designed to evaluate the effect of inhibiting aromatase activity in ovariectomized rats. Specifically, the effect on dendritic spine density and on the relative proportions of the different spine types was evaluated in pyramidal neurons located in the layer III of the prefrontal cortex. These changes were correlated with the performance of the experimental animals in an allocentric working memory test.

Materials and Methods

Subjects

Female Sprague-Dawley rats (n=48) weighing 250-300 gm were used in this study. The rats were housed at 25°C under a regular 12/12 h light/dark cycle, and with ad libitum access to water and food, and they were verified to complete three regular estrous cycles lasting 5 days.

Experimental design

The animals were assigned to one of four study groups: 1) a control group of bilaterally ovariectomized rats (Ovx; n=12); 2) another control group of bilaterally ovariectomized rats to which 1 ml of dimethylsulfoxide (DMSO) diluted 3% in saline solution was administered intraperitoneally (i.p., Oxv+Veh; n=12); 3) a further control group of rats studied in the proestrus stage (14:00 h) of their estrous cycle (Proestrus; n=12); and 4) an experimental group of bilaterally ovariectomized rats i.p. administered 1 mg/kg of Anastrozole [31,32] in 20 mg/ml DMSO diluted to 3% in saline solution (Ovx+Ans; n=12). The ovariectomized rats were first anesthetized with ketamine (60 mg/kg, i.m.) followed by 25 mg/kg of sodium pentobarbital (i.p.). The pharmacological treatments to the Oxv, Oxv+Veh and Oxv+Ans rats were applied on day 6 after ovariectomy.

From the first-to-seventh day after ovariectomy, the animals were all handled daily for 15 minutes in order to minimize the effects of stress. Furthermore, prior to the behavioral studies the animals had their food supply restricted to 85% of the daily intake to ensure their motivation for a food reward. In addition, they were not allowed any food for 24 h before behavioral testing. Access to water was always ad libitum. Six animals per group were used for behavioral testing, whilst morphological studies were performed by using the other six rats in each group (Figure 1).

Behavioral testing

The six animals from each of the study groups were challenged to resolve an allocentric working memory paradigm in a white, opaque acrylic "Y" maze that was situated in an illuminated room with

differential visuo-spatial signals. Each of the three arms of the maze could be closed with a sliding door, which were used in function of the test's needs. At the end of the adjacent arms of the Y, a food well below floor level contained cereal as a reward.

Before starting of the behavioral test, the animals were habituated to the maze by allowing them to explore the three arms of the maze freely for ten minutes. The rats were then withdrawn from the maze and immediately reintroduced into it, having placed several pieces of cereal on the floor of each of the three arms and within the wells. When the rat had eaten all the cereal or after a further ten minutes, the animal was returned to its home cage. Twelve trials were then carried out according to a delayed match-to-sample procedure in a single one-day session.

In each trial the rat was introduced into the Y maze, and a study phase was then followed by a delay period and a test phase. The rat was placed in the vertical arm of the Y maze for thirty seconds (introduction) before the starting arm was opened, and it was only allowed to enter the open arm and eat the cereal from the well (study phase). It was then returned to the starting arm for a period of ten seconds (delay period) before the sliding door of the starting arm was opened again, allowing entry into both of the upper arms of the Y maze so that the rat could choose either. However, only the arm that was baited during the study phase contained the reward. If the rat turned to the same arm as in the study phase then a "correct response" was registered in the trial (test phase: Figure 2). Each trial was separated by a thirty-second delay (interval period). To avoid egocentric tendencies in the resolution of the trials, a random design was used such that the rat had to turn to the right in six trials and it had to turn to the left in the other six trials.

Morphological studies

Rats from each group were anesthetized with ketamine (30 mg/kg, i.m.) and sodium pentobarbital (50 mg/kg, i.p.), and they were then perfused with 200 ml of a phosphate-buffered solution (pH 7.4, 0.01 M) containing sodium heparin (1000 IU/l) as an anticoagulant and procaine hydrochloride (1 g/l) as a vasodilator [33]. The rats were then perfused with 200 ml of a phosphate-buffered 4% formaldehyde fixative solution. Both solutions were perfused at a rate of 11.5 ml/min. The rat's brain was then removed and maintained for at least 48 h in 100 ml of fresh fixative solution. The bilateral blocks of prefrontal tissue were subsequently dissected out according to plates 7-11 of a brain atlas [34] and they were impregnated using a modified version of the Golgi technique [35]. Coronal slices (75 µm thick) were mounted on one slide per animal, and six third-layer pyramidal neurons of the pre-limbic/infralimbic prefrontal cortex from each of the six rats in

every group were analyzed. Pyramidal neurons were selected for spine counting based on three morphological criteria: (a) neurons had to be well impregnated; (b) neurons in which the bodies or branches selected for counting were obstructed by blood vessels, glia or heavy clusters of dendrites from neighboring impregnated cells were excluded; and (c) the apical arborization had to be fully impregnated and mainly intact. The dendritic spines were counted along a 50 μ m segment on each neuron in the middle section of a secondary dendrite protruding from the apical one (Figure 3, left panel). Sampling of the dendritic spines followed three morphological criteria: (a) belonging to a clearly distinguishable neuron found in layer three of the prefrontal cortex; (b) located on a secondary dendrite protruding from the middle third of the apical dendrite; (c) the dendritic segment selected for counting had to be in a homogenous plane of focus.

A reliability index was determined for dendritic spine counting in an initial double-blind study: number of agreements-number of disagreements/number of agreements. Once a minimum reliability of 0.95 was reached, dendritic spines from each of the groups were quantified using a “blind” procedure. The density and proportion of thin, mushroom, stubby and wide spines were determined according to previously established criteria [36-38]: thin spines were defined as those in which the neck diameter was less than the total spine length and the head diameter was only slightly greater than the neck diameter; stubby spines were defined as those in which the neck diameter was more than or equal to the spine length; mushroom spines were defined as those in which both the diameter and length of the neck was much smaller than the head diameter, and the neck was shorter than that of thin spines; wide spines, which closely resemble

stubby spines, were defined as those in which the total spine length was longer than the neck diameter (Figure 3, right panel). Spine counting was performed at 2,000X using a magnification changer in a light microscope coupled to an image analyzer (LAS 4.0).

Statistics

Behavioral data and spine density were analyzed by one-way ANOVA followed by the Tukey post hoc test, and the relative proportional density of each spine type was analyzed using one-way ANOVA followed by Bonferroni correction post hoc test.

Ethical considerations

All experimental procedures were designed to minimize the pain and discomfort of the experimental animals. The experimental protocol was approved by the Committee for Research Ethics of the Instituto Mexicano del Seguro Social (Mexico), and the experimental procedures were performed according to the NIH guidelines for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, 1996 revision).

Results

Allocentric working memory

The behavioral evaluation of allocentric working memory reflected significant variation between the four groups of animals studied ($F=27.546$, $p<0.0001$). The number of correct responses by the Proestrus rats in the Y maze was more than those recorded by the Ovx ($p<0.0001$), Ovx+Veh ($p<0.0001$) and Ovx+Ans ($p<0.0001$) animals. In addition, the Ovx+Ans rats made fewer correct responses than both the Ovx ($p<0.01$) and Ovx+Veh ($p<0.008$) animals. As expected, no significant differences were observed between the Ovx and Ovx+Veh groups (Figure 4).

Spine density

When the dendritic spine density was analyzed, significant differences were found between the groups studied ($F=16.893$,

$p < 0.0001$). Pyramidal neurons from layer III of the prefrontal cortex of Proestrus rats had a greater density of dendritic spines than Ovx ($p < 0.0001$), Ovx+Veh ($p < 0.0001$) and Ovx+Ans rats ($p < 0.04$). Similarly, there was a greater density of spines on neurons from Ovx+Ans animals than on those from the Ovx ($p < 0.01$) and Ovx+Veh ($p < 0.04$) groups. Again, there was no difference in spine density between the Ovx and Ovx+Veh rats (Figure 5).

Spine types

The proportion of thin spines ($F = 4.632$, $p < 0.01$), mushroom spines ($F = 12.989$, $p < 0.0001$) and filopodia ($F = 66.042$, $p < 0.0001$) varied between the distinct experimental groups of rats. The density of thin spines was greater in the Proestrus rats than in both the Ovx ($p < 0.03$) and Ovx+Ans ($p < 0.02$) groups, while there were no statistical differences between the Ovx, Ovx+Veh and Ovx+Ans rats. Similarly, a higher density of mushroom spines was evident in the Proestrus group when compared to the Ovx ($p < 0.0001$), Ovx+Veh ($p < 0.0001$) and Ovx+Ans rats ($p < 0.001$) and again, there were no differences in the density of mushroom spines between the Ovx, Ovx+Veh and Ovx+Ans groups. The proportions of stubby and wide spines did not vary significantly between the four groups studied. By contrast, there was a significantly higher proportion of filopodial structures in the Ovx+Ans animals than in the three other control groups: the Ovx ($p < 0.0001$), Ovx+Veh ($p < 0.0001$) and Proestrus animals ($p < 0.0001$; Table 1).

Spine type	Ovx	Ovx+Veh	Proestrus	Ovx+Ans
Thin	36.9 ± 1.8	37.8 ± 0.6	44.5 ± 1.4 ^a	38.8 ± 2.4 ^c
Mushroom	26.1 ± 0.7	26.1 ± 1.8	33.0 ± 1.1 ^{a b}	28.2 ± 0.8 ^c
Stubby	22.2 ± 0.7	20.4 ± 1.0	24.0 ± 1.2	24.5 ± 0.4
Wide	6.4 ± 1.1	5.3 ± 0.4	5.3 ± 0.3	6.2 ± 0.2

Filopodia	1.0 ± 0.2	0.2 ± 0.09	0.8 ± 0.2	5.9 ± 0.5 ^{a b c}
Mean ± SEM; ANOVA, Bonferroni; $p < 0.05$; a: vs. Ovx; b: vs. Ovx+Veh; c: vs. Proestrus				

Table 1: Density of the different spine types counted in pyramidal neurons from the layer III of the prefrontal cortex of female rats.

Discussion

Clinical [39,40] and experimental [23,41] studies have demonstrated an important regulatory role of estradiol on the morphological and functional characteristics of the neural substrate in the prefrontal cortex involved in the expression of verbal and spatial working memory [42,43]. In particular, the inhibition of estradiol synthesis dampens the ability to solve spatial working memory tests. Such an effect can be produced by interfering with testosterone aromatase activity, a phenomenon observed in women that receive anastrozole as a part of breast cancer therapy [28,44,45].

Anastrozole, as well as letrozole, are nonsteroidal testosterone aromatase inhibitors that bind reversibly to the aromatase enzyme, impeding the synthesis of estradiol otherwise resulting from testosterone aromatization [46,47]. Thus, besides breast cancer therapy, anastrozole has also been used as a clinical pharmacological approach aimed to counteract, through testosterone aromatase inhibition, other pathophysiological conditions in human beings, such as low-testosterone hypogonadism in elderly men [48]. In addition, anastrozole treatment has resulted in an experimental model of brain-synthesized estrogen insufficiency for different, *in vitro* [49,50] and *in vivo* [51-53], experimental designs.

On the other hand, changes in plasma estradiol levels during the estrus cycles may affect certain characteristics of learning and working memory [54,55] brain processes that require the processing and updating of information to produce short-term memory responses [56,57]. It is well known that peripheral plasma levels of estradiol, resulting from cyclic ovary steroid synthesis and secretion vary from basal values (less than 10 pg/ml) during estrus, and progressively increase through metaestrus and diestrus to achieve their maximal values on late proestrus (near around 40 pg/ml) [58]. When plasma levels of estradiol are higher, the performance of female rats in resolving spatial working memory tests is preferentially based on allocentric strategies rather than egocentric strategies [59], a result of a switching activity in the prefrontal cortex that foments either allocentric or egocentric performance [60].

Moreover, estradiol therapy improves the deficient performance of ovariectomized rats in prefrontal cortex dependent allocentric working memory tests, as well as eliciting plastic changes and an increase in dendritic spine density, mainly due to the higher proportion of thin dendritic spines in layer III pyramidal neurons of the prefrontal cortex [61,62]. These neurons are involved in determining whether allocentric or egocentric strategies are employed to solve working memory tests, as well as in processing the sensory/motor memory information required for the memory fields to adopt the adequate working memory strategy [63-65]. Here, ovariectomized rats resolved the working memory task inefficiently and they did so at a probabilistic level of chance (50%). However, ovariectomized rats that received anastrozole performed below 50% efficiency and they were even less efficient than the control ovariectomized rats. These findings of significantly fewer correct responses when ovariectomized rats received anastrozole in the allocentric spatial working memory test, suggests that the estradiol

synthesized in brain structures, including the prefrontal cortex, may fulfill a relevant role in the neural substrates involved in cognitive functions. Indeed, estrogenic activity has been proposed to affect synaptic plasticity and the proportions of dendritic spines, thereby influencing cognitive performance. Such a phenomenon may be particularly relevant if driven by estradiol synthesized locally in specific brain structures rather than being supplied from that synthesized in the ovary [24,66].

There is evidence that the plasticity of dendritic spine synapses driven by the estrogen produced through brain aromatase activity may have different functional characteristics depending on the specific brain structures where they were synthesized, in female rats [19,67,68]. In addition, the effects of aromatase inhibition on dendritic spine plasticity may change in different experimental conditions. Thus, while inhibiting aromatase with letrozole in cycling rats did not affect synaptic spine density on prefrontal cortex pyramidal neurons such treatment did significantly reduce the number of dendritic spine-mediated synapses. Moreover, aromatase inhibition down-regulated synaptophysin and spinophilin expression in the hippocampus of both cycling and ovariectomized rats [30], and in hippocampal cell cultures [68].

In the present work, dendritic spine density on layer III pyramidal neurons in the prefrontal cortex increased significantly as a result of anastrozole-induced inhibition of brain aromatase activity in the absence of a gonadal source of estrogen in the brain. This increase was associated with a higher proportion of filopodia. According to earlier pioneering studies [69], increased dendritic spine density would lead to better synaptic efficiency. Thus, the higher proportion of filopodia may reflect the initial steps of neuronal activation and the development of neural processes during de novo dendritic spine formation [70-73]. However, recent studies suggested that although mature spines could develop from filopodial structures in adult rodents, these might be inactive [74]. The formation of inactive spines would be consistent with our findings, as despite the increase in dendritic spine density in ovariectomized females that received anastrozole, they performed worse in the working memory task. Thus, while the increase in dendritic spines observed would suggest a compensatory response, our behavioral findings show that these morphological changes seem not to provoke functional compensation on neural substrates in the prefrontal cortex when the modulatory activity of estradiol is absent.

Thin spines have been associated with learning capabilities whilst mushroom spines have been related with memory storage [75,76]. Synaptic stimulation through both stubby and wide spines has been suggested to regulate neuron excitability [36,77]. It is noteworthy that the proportional density of any spine type was unaltered by anastrozole administration to the ovariectomized animals. This is particularly relevant given the increase in dendritic spine density and the enhanced working memory impairment. On the one hand, the homogenous increase in spine types would account for the “stable” postsynaptic translation of synaptic information and on the other hand, the non-predominance of a specific type of spine suggests that the lack of E2 has a homogeneous impact on spine dynamics.

Thus, neural processes appear to be altered by the loss of the estrogenic activity resulting from estradiol synthesis by the neural substrates in the prefrontal cortex that drives cognitive activity, a phenomenon that merits further research.

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