Morphological Changes Induced by the Absence of Ovarian Hormones in Nucleus Accumbens of Ovariectomized Rats

Alma Rosa Quiñones[1], Israel Camacho-Abrego[2], Nancy Antonia Rivera[2], Gonzalo Flores[2] and Ofir Picazo[*,1]

[1]*Escuela Superior de Medicina del I.P.N., Plan de San Luis y Diaz Mirón, Col. Sto. Tomás, México D.F. 11340, México*

[2]*Laboratorio de Neuropsiquiatría, Instituto de Fisiología, Universidad Autónoma de Puebla. 14 Sur 6301, CP: 72570, Puebla, México*

Abstract: Ovariectomy (OVX) has been widely used to explore the effect of ovarian hormones on brain regions. It has been observed that estrogen in OVX rats increases the dopaminergic function in nucleus accumbens (NAcc). To determine whether absence or presence of estradiol changes the dendritic structure of some dopaminergic neurons, we investigated the consequences of a daily injection for 2 weeks of 17-beta estradiol (E2) on the dendritic morphology of NAcc neurons in two groups of rats, immediately or 10 weeks administered after OVX. The structure of dendrites was measured by using the Golgi-Cox procedure followed by a Sholl analysis. We found a reduction in the number of spiny dendrites of the medium spiny neurons of the NAcc in animals in which E2 was administrated without delay after OVX. However, when the same treatment was administered 10 weeks after OVX, not any difference was observed. Independently of the hormone treatment, rats from the group of 10 weeks after OVX showed a reduction in the number of spiny dendrites of the medium spiny neurons when compared with their counterparts at 2 weeks. Data suggest that estrogens or their absence regulate synaptogenesis in the NAcc.

INTRODUCTION

The decline of the ovarian function occurring in aged females has been related to many somatic and psychological disturbances [1-5], changes in several neurotransmitters and their receptors [6-9], and variations in the fine neuronal structures in several brain regions [10-12]. Thus, while the ovarian hormones estrogen and progesterone are able to modify the dendritic spine density and the morphology of neurons from various brain regions [13-15], their absence induced through an ovariectomy (OVX), can affect the dendritic arbor of such structures [12-14, 16, 17]. However, the influence of estrogens on dendritic structure at NAcc has not yet been documented.

Theoretically, the gradual reduction of steroid hormones characterizing the menopausal period can be mimicked by the removal of ovaries. This manipulation has been proposed as a model for studying post-menopausal osteoporosis in humans [18-20] and recently has also been used to show that some behavioral symptoms associated with human post-menopause can be studied in rats OVX 12 weeks previously [4]. The current study utilized this same model to explore possible changes in the neuronal morphology of NAcc, influenced by the absence of ovarian hormones. These results were compared to rats treated with E2 for two weeks, either immediately or ten weeks after OVX. The Golgi-Cox stain method and Sholl analysis were employed to determine dendritic length, neuronal branching and spine density of spiny medium neurons of the NAcc.

*Address correspondence to this author at the Escuela Superior de Medicina del I.P.N., Plan de San Luis y Diaz Mirón, Col. Sto. Tomás, México D.F. 11340, México; Tel: (xx) 55 57296300, Ext. 62761; Fax, Ext. 62806;
E-mail: rifo99mx@yahoo.com.mx, rifo99@hotmail.com

MATERIALS AND METHODOLOGY

Forty female Wistar rats were obtained from our animal facility (Escuela Superior de Medicina-IPN, ESM-IPN). All rats weighed between 225 and 250 g at approximately 3 months of age. Animals were housed in pairs and maintained in a temperature and humidity controlled environment, with free access to food and water. All surgical procedures described in this study were approved by the ESM-IPN Animal Care Committee and met governmental guidelines (Mexican Council for Animal Care, Norma Oficial Mexicana NOM-062-ZOO-1999). All efforts were made to minimize both animal suffering and to reduce the number of animals used. All animals included in this study were ovariectomized through a dorsal incision under 2,2,2, tribromoethanol anesthesia (0.2 g/kg, i.p.). The complete extraction of the ovaries was corroborated by visual inspection. Post-surgical care included administration of the antibiotic Baytril (0.03 ml/animal, SC) and analgesic banamine (0.03 ml/animal, SC). Subsequent to surgery, all animals were assigned to two groups, the first group received immediately a daily subcutaneous injection of corn oil (1 ml/kg, n=10) or 17-β estradiol benzoate (50 µg/kg n=10) during two weeks after OVX. The second group received the same treatment ten weeks after the surgery [(10-w-OVX) (n=10, each for vehicle and E2)]. Doses and treatments were selected according to previous reports [21]. All injections were done between 800–1000 h each day.

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One day after the last E2 injection, rats were deeply anesthetized with sodium pentobarbital (60 mg/kg body weight, ip) and perfused intracardially with 0.9 % saline solution. The brains were rapidly removed and processed by the modified Golgi-Cox method [22]. Briefly, the brains were stored in the dark first in the Golgi-Cox solution for 14 days and then in a 30% sucrose solution for 3 days. The brains were then blocked and sliced coronally in 200 µm-thick sections at the level of the NAcc using a vibratome (Campden Instrument, MA752, Leicester, England). The sections were collected on clean, 2% gelatin-coated glass microscope slides (4 sections/slide). The mounted tissue was rinsed in distilled water and then placed in a bath of ammonium hydroxide for 30 min in the dark. After rinsing, the sections were then immersed in the dark in Kodak Film Fixer for 30 min and subsequently washed with distilled water, dehydrated and cleared in successive baths of 50% (1 min), 70% (1 min), 95% (1 min) and 100% (2x5 min) alcohol followed by 15 min in a xylene solution. The slides were covered with balsam resinous medium [23, 24].

Medium spiny neurons of the NAcc (plate 9 - 12) of Paxinos and Watson Atlas [25] were selected for this study. For each animal, five neurons from both the left and right NAcc were drawn using camera lucida at a magnification of 250X (DMLS, Leica Microscope) by a trained observer, who was blind to the experimental conditions [26]. Medium spiny neurons from the NAcc were identified by soma size and dendritic extension, as described by Robinson and Kolb [27] and Flores *et al*., [22]. The criteria used to select neurons for reconstruction have been described in details elsewhere [22, 28-30]. Briefly, only complete, fully impregnated pyramidal neurons with no apparent truncation of basal dendritic arbor were included in our analyses; terminals were positively identified by their characteristic conical shape. Our analyses were performed on basal dendrites of pyramidal neurons as they are parallel to the coronal plane. A student blind to the experimental procedures was assigned to observe, identify and reconstruct the three-dimensional dendritic tree by drawing each neuron in a two dimensional plane. The dendritic tree was studied by Sholl analysis as follows: a transparent grid with concentric rings, equivalent to 10 µm apart, was placed over the dendritic drawing and the number of ring intersections were used to estimate total dendritic length [23, 31]. Another estimate of dendritic arborization is the total number of dendritic branches (branching indicated by bifurcation) which were counted at each order away from the cell body or dendritic shaft. Finally, the density of dendritic spines was estimated by randomly selecting high-magnification (1,000X) tracings of a 10-µm-long terminal segment of the dendritic branch; in most cases the length of the selected distal branch exceeded 40 µm. Estimates of spine density were generated by counting the number of visible spines along the branch segment and expressing the data as number of spines/10 µm.

Mean values from NAcc of each animal were treated as a single measurement for the data analysis. Data on dendritic length and spine densities were analyzed by two-way ANOVA, followed by Newman-Keuls test for post-hoc comparisons, with E2 treatment and time after OVX as independent factors. Data from length per branch order were analyzed using a two-way ANOVA, followed by Bonferroni test for post-hoc comparisons, with E2 administration and branch order as independent factors ($P<0.05$ was considered significant).

RESULTS

The morphological analysis presented here is based on 400 NAcc medium spiny neurons from 40 animals and the E2 effects on dendritic morphology in the NAcc from ovariectomized animals are illustrated in Fig. (**1**). The neuronal dendritic morphology of NAcc neurons was measured by Golgi-Cox stain and dendritic length for each branching order, spine density and total dendritic length were obtained as reported previously [22, 24, 28-30]. The Golgi-Cox impregnation procedure clearly filled the dendritic shafts and the spines of neurons from Nacc (Fig. **1A**).

The two-way ANOVA analysis revealed a significant effect of time after OVX ($F_{1, 35}$=13, $P < 0.001$) and interaction of E2 and time ($F_{1, 35}$=6.1, $P = 0.01$) in dendritic spine density of medium spiny neurons of the NAcc (Fig. **1B**). Post-hoc test revealed that dendritic spine density was significantly lower in the medium spiny neurons of the NAcc from the immediately-OVX rats with E2 administration compared to their corresponding vehicle group ($P < 0.01$). Interestingly, the E2 administration in the 10-w-OVX rats did not produce any difference (Fig. **1B**). The analysis between vehicle groups revealed that dendritic spine density was significantly lower in the medium spiny neurons of the NAcc from 10-w-OVX rats with vehicle compared to immediately-OVX rats with vehicle as well ($P < 0.001$) (Fig. **1B**). The total dendritic length of medium spiny NAcc neurons (Two-way ANOVA, between E2: $F_{1, 35}$=1.9, $P = 0.1$; time post-OVX: $F_{1, 35}$= 0.5, $P = 0.1$ and interaction E2 and time post-OVX $F_{1, 35}$= 0.45, $P = 0.5$) revealed no difference between groups (Fig. **1C**). Two-way ANOVA for the analysis of length per branch order of medium spiny NAcc neurons from immediately-OVX rats with E2, revealed a significant effect of branch order ($F_{1, 35}$=88, $P < 0.01$), as well as an interaction of this factor with E2 administration ($F_{1, 35}$=2.05, $P = 0.04$) (Fig. **1D**). Post-hoc test revealed that the dendritic length at the level of the third order decreased in the immediately-OVX rats with E2 administration compared to corresponding vehicle groups ($P < 0.05$) (Fig. **1D**). Finally, two-way ANOVA for the analysis of length per branch order of medium spiny NAcc neurons from 10-w-OVX rats with E2 did not show any differences (Fig. **1E**).

DISCUSSION

These data evidence that E2 induces a differential effect, which is dependent on the time of hormone administration after OVX. Thus, a reduction in the number of spiny dendrites of the medium neurons of the NAcc in the animals immediately treated (for two weeks) with the hormone after OVX, compared to the respective control, was observed. In contrast, no difference was found with the same treatment administered 10 weeks after the surgery. In addition, rats from 10-w-OVX control group showed a reduction in the number of spiny dendrites analyzed in comparison to immediately-OVX control group. Data suggest that estrogens or their absence regulate synaptogenesis in the NAcc.

The mechanism by which E2 induces changes in dendritic architecture at NAcc is not clear. However, both dendritic growth and spiny density on dendrites seem to be

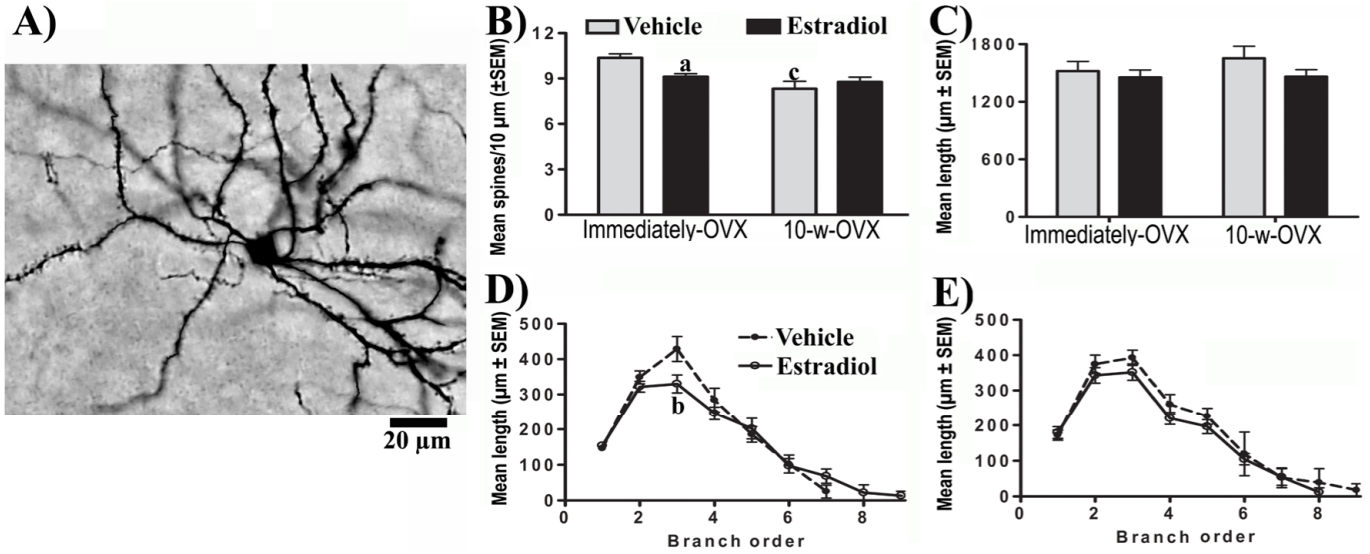

Fig. (1). Analysis of NAcc medium spine neurons in rats with estrogenic treatment for two weeks at two different post-OVX times, immediately after- and 10 weeks after-OVX (n = 10 animals per group). (**A**) Golgi-Cox-impregnated nucleus accumbens neuron from a vehicle treated animal immediately after OVX. (**B**) Spine density decreased in immediately-OVX rats with E2 compared to their corresponding vehicle group ($^{a}P < 0.01$). The E2 administration in the 10-w-OVX rats did not produce any difference. Interestingly, 10-w-OVX group with vehicle also showed significant decrease in spine density compared to immediately-OVX rats with vehicle ($^{c}P < 0.001$). (**C**) Total dendritic length analysis revealed no differences among groups. (**D**) Length of branch order of the rats treated with E2 immediately after OVX. The analysis revealed that the dendritic length at the level of the third order decreased in the immediately-OVX rats with E2 administration compared to corresponding vehicle group ($^{b}P < 0.05$). (**E**) Length of branch order of the 10-w-OVX rats with E2. The analysis of length per branch order of medium spiny NAcc neurons from 10-w-OVX rats with E2 did not show any difference.

related with its degree of connectivity and afferent activity [32]. For example, loss of principal afferents to spiny neurons during early development produces alterations in dendritic spines [33-38], while the decreased afferent activity by *in vivo* NMDA receptors blockade, reduces dendritic growth and branching of cerebellar and tectal neurons [39-41]. It is known that NAcc medium spiny neurons receive glutamatergic inputs from prefrontal cortex and hippocampus [42-44] and recent reports suggest that OVX produces a decrease in the dendritic spines density at the level of hippocampus and prefrontal cortex. Therefore, a decrease of the glutamatergic inputs to medium spiny neurons of the NAcc may results in a decrease in the number of spines [32] as observed in 10-week control group in comparison to 2-week control group.

Data regarding the influence of ovarian hormones on the structure of neurons are contradictory. Thus, OVX results in a decrease in the number of dendritic spines in hippocampus and prefrontal cortex [12]. Contrarily, proteins MAP and Tau, involved in the development of dendrites and axons respectively, do not change in frontal cortex as a consequence of E2 or P treatment [45], or due to hormonal variations along the estrous cycle [46]. Similarly, density of dendritic spines in the hippocampal CA1 stratum radiatum, seems to be not related with the high levels of estrogens reached during proestrous [47]. These data support the idea that estrogenic actions on dendritic architecture is regulated by several factors including the time elapsed after OVX and the connectivity and afferent activity of some particular brain region. In this sense, removal of ovaries can increase the dendritic branch number and spine density of pyramidal cells from parietal cortex observed four months after an OVX [48], while an estrogenic treatment similar to used in the current study, decreases the number of branches of dendrites of layer II/III pyramidal cells in the entorhinal cortex [49]).

As mentioned, long-term removal of ovaries could be useful to study some characteristics of human post-menopause. Thus, the absence of an effect of E2 therapy in 10-week rats seems to be comparable to some clinical data showing no benefit and even potential hazards of E2 treatment [50-52]. This idea has been supported by the observation that similar hormone levels act differently on the brain of a younger individual compared with an older one [10, 53, 54] and by the fact that E2 applied 30 days, but no 10 days, after OVX does not produce any effect on cell density in the compact zone of the substantia nigra [55]. Similarly, E2 increases dendritic spine density in CA1 pyramidal cells of young female rats but such an effect is attenuated in aged monkeys [56], highlighting the role of the estrogenic milieu on synaptogenesis and supporting the hypothesis that long-term OVX rats could be a useful model to study some disorders associated to human menopause.

CONCLUSION

This study shows that both OVX and estrogenic replacement short- or long-term after this surgery can produce different actions on neuronal architecture in NAcc of rats. Due to the consequences of these changes, time frame after cessation of ovarian activity in women must be taken into account when beginning a hormone replacement therapy.

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