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INFLUENCE OF ESTROGEN ON THE CEREBELLAR CORTEX OF MALE RATS

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Effects of estrogen in the brain regions which are not directly related to neuroendocrine functions are not fully known. Therefore we investigated the long term effects on the cerebellar cortex of a neonatally (3rd day) administered single dose (1 mg) of estrogen. Golgi impregnated and paraffin sections (Bielschowsky, Klûver-Barerra and basic fuchsin-alcian- blue) of cerebella of 10 treated adult (365 days old) male rats and of 10 matched controls were studied. Simultaneous to the morphological analysis of the cerebellar cortex, stereological methods were applied.

In treated rats the Purkinje neurons had a more developed dendritic arborisation with numerous spines, but had significantly decreased ($p<0.001$) cellular body volumes (14×10^{-3} mm^3) in comparison to controls (27×10^{-3} mm^3). In treated animals the molecular layer thickness (148.94 μm) of the cerebellar cortex was significantly decreased ($p<0.001$) compared to controls (514.52 μm), and the thickness of the granular layer (372.35 μm) was significantly ($p<0.001$) increased compared to controls (270.80 μm). In treated rats the number of neurons in the granular layer was 338.87 mm^2 (controls 118.81 mm^2) and in the molecular layer was 22.86 mm^2 (controls 55.23 mm^2). Our results indicate significant and long term effects of a single dose of estrogen (administered in the neonatal period) on the cerebellar cortex of male rats.

Key words: estrogen, rat, cerebellar cortex, Purkinje cells, morphometry, morphology

INTRODUCTION

Since the article of Raisman and Field (1973) the action of sex steroids on the brain was extensively studied. In the classical concept estradiol, as well as progesterone, is known to be a sex steroid and acts on brain tissues through

intranuclear receptor-mediated mechanisms and regulates reproductive behavior (Arnold and Gorski, 1984; Tsutsui *et al.,* 2000).

Previous studies have failed to demonstrate estrogen-concentrating cells or estrogen receptor (ER) mRNA in the adult rat cerebellum (Pfaff and Keiner, 1973; Simerly *et al.,* 1990), but recent studies suggested that estradiol during cerebellar development can influence the Purkinje cells via ERß (Shughrue *et al.,* 2001; Price and Handa, 2000; Shughrue and Merchenthaler, 2001). In addition, developing Purkinje cells may also produce estradiol (Ukena *et al.,* 1999). The Purkinje cells, which possess steroidogenic enzymes and produce sex steroids in a variety of vertebrates, including mammals (Ukena *et al.,* 1999; Sakamoto *et al.,* 2001) are a major site for neurosteroid formation (Sakamoto *et al.,* 2003). During neonatal life in the cerebellar cortex of the rat marked morphological changes occur (Altman, 1972). The knowledge of long term changes in the morphology of neurons influenced by estrogen could be significant in the understanding of neuroplasticity.

The aim of our morphological and morphometric study is to investigate the long term effects of neonatally administered estrogen on the cerebellar cortex of male rats. We analyzed the effects of estrogen on Purkinje cells (their soma, dendritic tree and spines), as well as on the thickness and number of neurons of the molecular and granular layer.

MATERIAL AND METHODS

The morphology and morphometry of the cerebellar cortical layers and of the Purkinje cells were investigated in 10 control and 10 treated male Wistar rats housed in accordance with international guidelines on the ethical use of animals. Animals were housed at constant room temperature (food and water *ad libitum*). The rats were neonatally (3 days of age) treated with a single dose (1 mg) of estradiol dipropionate (E_2) in oil solution (GALENIKA, Belgrade) and were sacrificed on the 365th day of life after deep ether anesthesia, by intracardiac perfusion of 10 % neutral formalin. Brains were removed and after four weeks of fixation in 10 % neutral formalin were divided into smaller blocks. Some of these blocks were impregnated by a modification of the Golgi - Kopsch method (Drekić and Malobabić, 1987) wille others were processed for routine paraffin sections.

In brief, for the Golgi impregnation the tissue blocks were kept in 2% potassium dichromate solution at 37°C for 1-2 days. The solution was changed daily and after that the tissue blocks were left for 3 days in fresh 2.5 % silver nitrate solution at 37°C, and then in 96% ethanol for 24 hours. Dehydration was completed in 100 % ethanol in which the impregnated blocks were stored. After encasing the blocks in liquid paraffin (56 °C) and leaving them for 10-20 minutes at room temperature, they were cut into serial frontal (140 μm thick) sections which were soaked into xylol for at least 10 minutes and then mounted. The morphology of neurons and of their dendritic arborisations was investigated on impregnated Purkinje cells on selected complete Golgi sections of male rat cerebellar cortex.

The other tissue blocks, after routine processing for paraffin embedding, were serially cut into 5 μm thick frontal sections which were stained with

Bielschowsky, Klüver-Barerra and basic fuchsin-alcian- blue method. The sections were mounted with Canada balsam.

The thickness of molecular and of granular layer of cerebellar cortex, as well as the dendritic arborizations and the volume of the Purkinje cell soma were determined on Klüver - Barrera stained sections.

For linear measurements and for stereological analysis the ocular graticule was used at a magnification of 190 x. The volume of the perikaryon of Purkinje cells was calculated according to the formula for a rotatory ellipsoid conus (1/6 • 3.14 •d^2 • D), in which formula d is the smaller, and D is the larger diameter of neuronal soma. During the estimation of the number of neurons only the nucleated neurons were counted on every second section using the stereological test system Weibel- A 100 (Kalisnik, 1982). Results, in addition to the methods of descriptive statistics were analised by the Students' t-test.

RESULTS

Control animals

Morphology: In control male rats the soma and dendrites of Purkinje cells were well developed. In general the dendritic spines were rare and more present on the distal dendrites (Fig. 1A and 1 C and Fig. 3A).

Morphometry: The volume of the soma of Purkinje cells was $27x10^{-3} \pm 1.25$ mm^3. The thickness of the molecular layer was 514.52 ± 1.25 μm, and of the granular layer was 270.80 ± 0.51 μm (Fig. 2A and Fig. 3A). The number of neurons in the molecular layer was 55.23 mm^2 ± 1.47 and in the granular layer was 118.81 mm^2 ± 1.53.

Treated animals

Morphology: In treated rats Purkinje cells were more darkly impregnated and their bodies had a more pyramidal or piriform shape than the controls. In contrast to the controls, their thicker and more arborisied dendrites had numerous spines and contacts with other Purkinje cells (Fig. 1B and 1D).

Morphometry: In treated rats the volume of the soma of Purkinje cells was $14 \times 10^{-3} \pm 1.50$ mm^3 that was significantly ($p<0.001$) decreased than in the controls. The molecular layer of cerebellum in treated rats was significantly ($p<0.001$) thinner (148.94 μm ± 0.75) and granular layer was significantly ($p<0.001$) thicker (372.35 μm ± 0.53) than in control animals.

The number of neurons in the molecular layer of treated rats was 22.86 mm^2 ± 0.51, which was significantly ($p<0.001$) compired to the controls (Fig. 3B). The number of neurons in the granular layer was 338.87 ± 1.45 mm^2 which was significantly ($p<0.001$) increased compared to the controls.

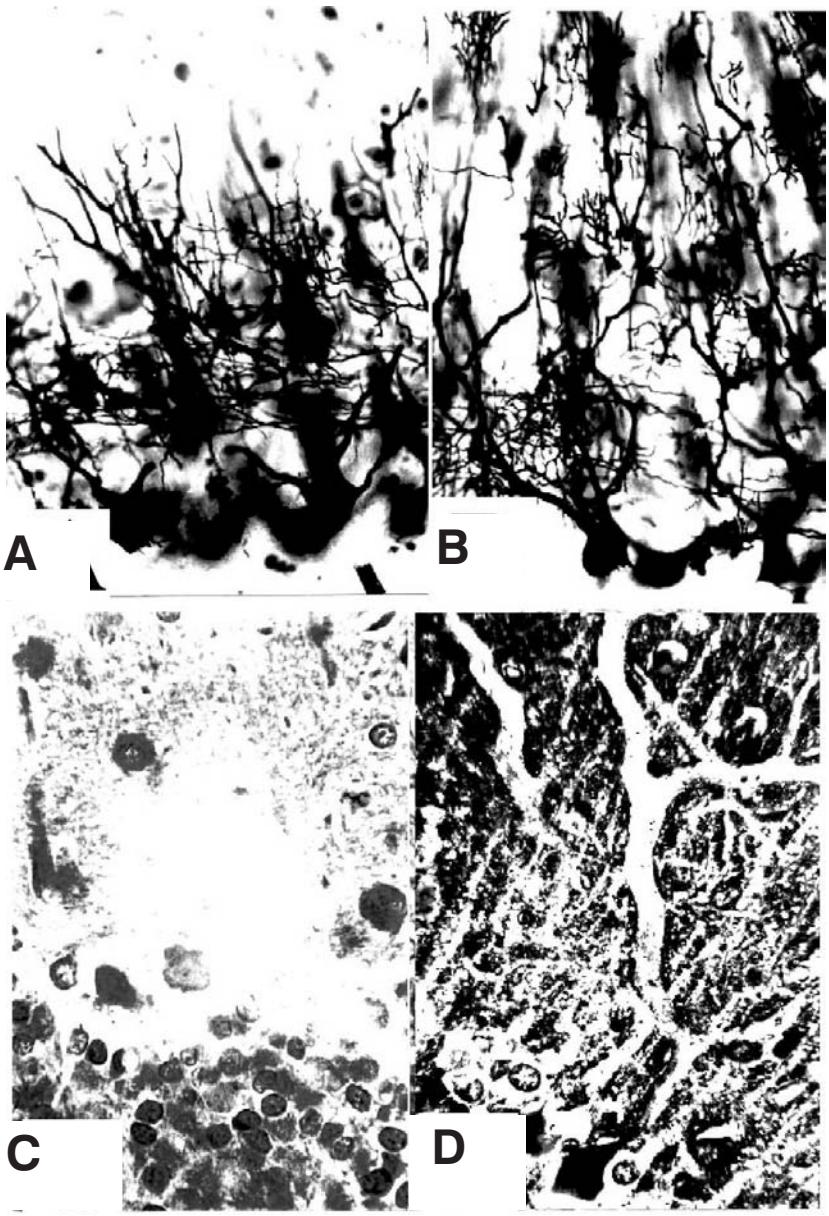

Figure 1. A and C: Moderate dendritic arbors of Purkinje cells in control rats (A - Golgi method; C- basic - fuchsin). B and D: Well developed dendritic arbors of Purkinje cells in treated rats (B - Golgi method; D- basic - fuchsin). 360 X)

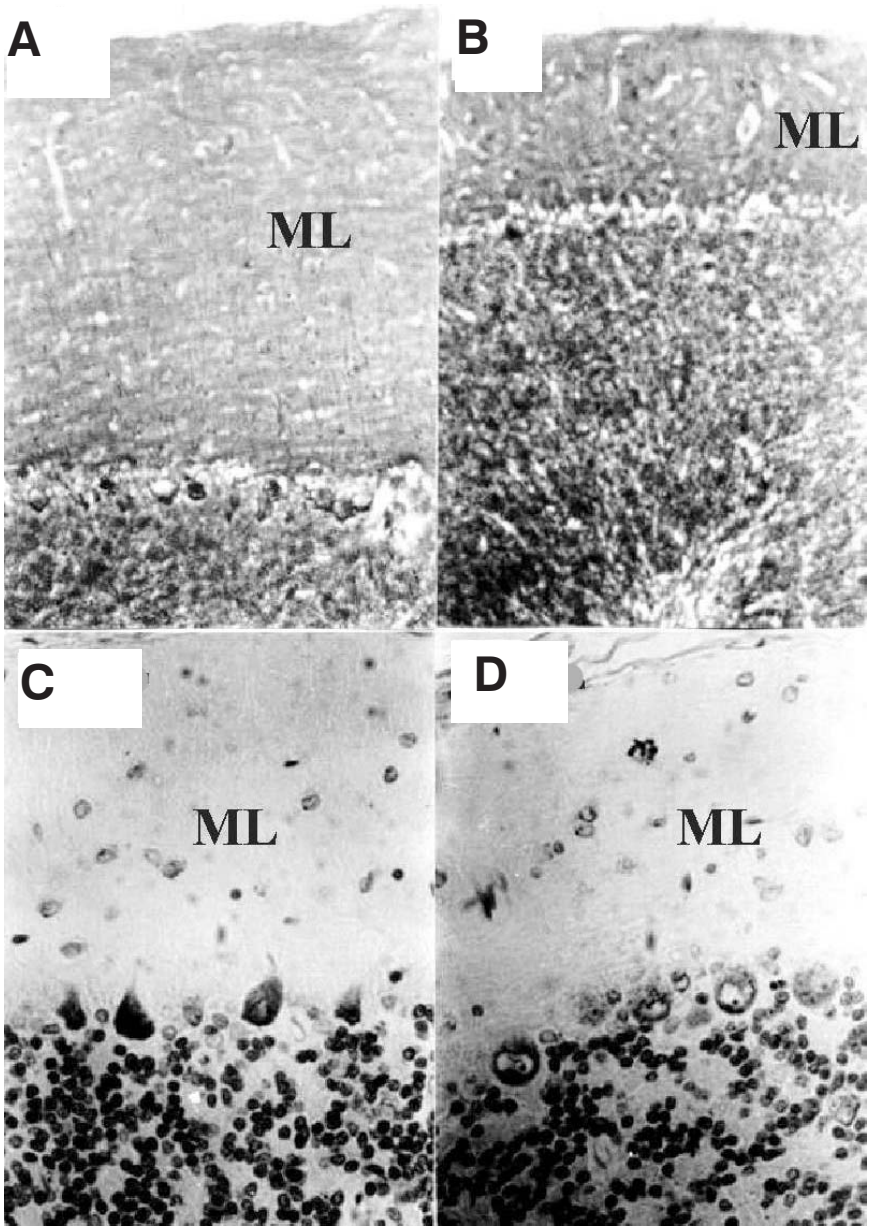

Figure 2. Thicker molecular layer (SL) in controls (A and C) than in treated rats (B and D). The granular layer (SG) is thinner in controls (A and C) than in treated rats. (A and B - Bielschowsky stain; C and D- Klûver –Barrera method; 144 X)

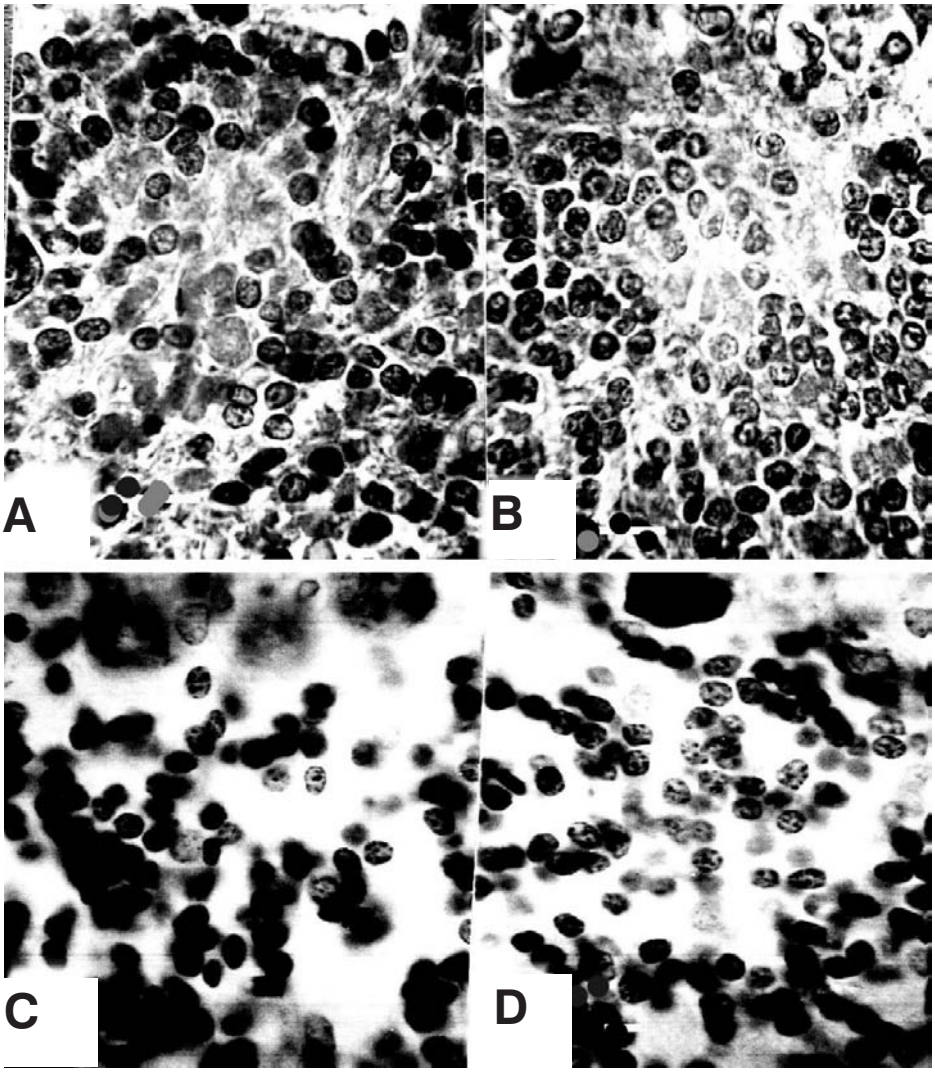

Figure 3. Increased number of neurons in granular layer (SG) of treated rats (B) than in controls (A). (basic fuchsin; 1440 X)

DISCUSSION

This study clearly showed the long term (after one year) effect of neonatally administered E_2 on the cerebellar cortex of male rats. Dramatic morphological changes caused by E_2 were found on the Purkinje cells (including their dendritic tree) and in the cerebellar cortical layers.

E_2 induced the presence of more spines on dendrites of Purkinje neurons and a significant increase in the thickness of the granular layer, accompanied with a significant decrease in the thickness of the molecular layer and size of Purkinje cell bodies. Whether this resulted solely from the direct influence of E_2 on the cerebellar cortex, or could be at least partially, ascribed to the changes in other functional systems (mossy and climbing fibers for example) requires further studies. Purkinje cells highly express mRNA encoding for P450 aromatase, a key enzyme of estrogen formation in the neonatal rat at 8 days of age and the transient estrogen formation in the rat cerebellum may occur during neonatal life (Sakamoto *et al.,* 2003).

Our finding of better developed Purkinje cells dendritic tree with more spines confirms that estradiol may act directly to promote the dendritic growth and spinogenesis during cerebellar development through ERβ-mediated mechanisms (Price and Handa, 2000). The transcripts for ER subtypes α and β in the cerebellum were co-expressed in the neonatal rat, but no ER α transcripts were detectable in adult rats. However, for ER β transcripts there was not significant difference between neonatal and adult cerebella (Zhone *et al.,* 2001).

Estradiol increased both, the dendritic growth of Purkinje cells, and the density of their dendritic spines in newborn rats (Sakamoto *et al.,* 2003). These effects were inhibited by the ER antagonist tamoxifen (Webb *et al.,* 1995). However, estradiol might act on Purkinje cells via non nuclear ERs (Smith *et al.,* 1988), or through possible nongenomic action via ERα in the brain. Regulators of Purkinje cell dendrite and spine development are highly expressed in the developing cerebellum and are critical for proper development of Purkinje and granule cells (Bates *et al.,* 1999; McEwen *et al.,* 2001). In our study is confirmed that estradiol contributes to the growth of Purkinje cells of the rat during neonatal life (Sakamoto *et al.,* 2003). It has been reported that estradiol promotes synaptogenesis and spinogenesis in other brain regions, such as the hippocampus (Woolley and McEwen, 1994) hypothalamus (Langub *et al.,* 1994), amygdala (Drekić *et al.,* 1990; 1995) and parietal cortex (Drekić *et al.,* 1992).

The antiestrogen (ER antagonist) tamoxifen blocks transcriptional activation of ERβ in the developing Purkinje cell with reversed effects on its morphology, resulting in sparse dendrites and lacking dendritic spines (Webb *et al.,* 1995). The absence of almost all the dendritic spine-like structures in Purkinje cells after the treatment with tamoxifen suggests that estradiol acts on Purkinje cells via ERβ, to induce not only dendritic growth but also dendritic spine formation (Webb *et al*., 1995). The expression of P450 aromatase mRNA in the cerebellum is restricted to Purkinje cells and external granule cells in neonatal rats (Tsutsui *et al.,* 2001), which can be related to our findings of increased thickness of the granular layer in treated rats.

Our findings indicate increased synaptogenesis in treated animals in the form of more developed Purkinje cells dendritic arborization and more frequent presence of spines on them. This was accompanied by decreased thickness of the molecular layer which houses the investigated Purkinje cells dendrites.

In conclusion, our study indicates the long term effects of E_2 on the cerebellar cortex of male rats, observed one year after its neonatal administration.

These long term effects are expressed as changes in morphology and size of Purkinje cells, as well as changes in the thickness of cerebellar granular and molecular layers. Because our results are not expressed in a simple or linear manner in all investigated structures, it is obvious that the plastic changes of the cerebellar cortex caused by estrogen are very complex.

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DUGOTRAJNI UTICAJ ESTROGENA NA KORU MALOG MOZGA MUŽJAKA PACOVA

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SADRŽAJ

Dugotrajni efekat jedne doze (1 mg) estradiol dipropionata (E2) proučavan je na kori malog mozga mužjaka pacova tretiranih u neonatalnom (trećeg dana) i žrtvovanih u adultnom (365 dana) periodu života. Na Goldži impregniraniom preparatima i parafinskim rezovima (Bielschowsky, Klûver-Barerra i baznim fuksinom i alcian-plavim) kore (molekularni sloj, Purkinjeove ćelije i granularni sloj) malog mozga proučavana je morfologija i stereološki parametri.

U tretiranih mužjaka grananje dendrita Purkinjeovih ćelija bilo je značajno uvećano, a takođe je značajno bila smanjena i zapremina tela Purkinjeovih ćelija ($14x10^{-3}$ mm^3) u poređenju sa odgovarajućim kontrolama (27×10^{-3} mm^3). Takođe je kod tretiranih životinja bio povećan broj spina (sinapsi) na dendritima Purkinjevih ćelija. Molekularni sloj je bio značajno stanjen, a granularni sloj značajno zadebljao u poređenju sa odgovarajućim kontrolama. Prema našim nalazima estrogen dat u neonatalnom periodu života verovatno je preko genomskog delovanja izazvao trajne morfološke promene u slojevima kore malog mozga mužjaka pacova žrtvovanih u adultnom periodu života. Ovo ukazuje na značajan produženi efekat jedne doze estrogena na koru malog mozga mužjaka pacova.