Prenatal corticosterone influences the trajectory of neuronal development, delaying or accelerating aspects of the Purkinje cell differentiation

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Summary. The nervous system developmental programs proceed in orderly fashion following strict timetables. However, the mechanisms regulating developmental timing remain largely unknown.

Increases or decreases in glucocorticoids in the fetal brain can be detrimental. We present evidence supporting that corticosterone forwards the migration of cerebellar granule neurons when applied acutely during pregnancy. This change in developmental *tempo* enhances dendritic growth of Purkinje neurons, increases the nuclear area, accelerates perinucleolar rosette appearance and decreases the development of Nissl bodies. Our observations thus support that forwarding the occurrence of developmental events does not always arrest neuronal growth, as some heterochronic developmental models imply. We suggest that prenatal glucocorticoids alter the trajectory of Purkinje neurons development soon after birth. These changes could represent a transient condition or could produce medium or long-term later consequences. More studies are needed to evaluate these intriguing possibilities.

Key words: Purkinje neurons, Differentiation, Prenatal glucocorticoids, Ultrastructure, Golgi-Kopsch technique

Introduction

The use of corticoids to promote fetal lung maturity of preterm infants has become an accepted practice within modern obstetrics (Crowley et al., 1990). There is increasing evidence that corticosteroids not only reduce the risk of respiratory distress syndrome and neonatal mortality, but also reduce the risk of neonatal neurological morbidity (Gray et al., 2001). However, negative long-term effects of prenatal steroids on infant

development have been documented, and there are few suggestions of adverse consequences of perinatal glucocorticoid treatment in the short term subsequent childhood (Matthews, 2000; Seckl, 2000).

The development of the nervous system is a complex process that involves a wide variety of genetic factors, endocrine *milieu*, cell interactions and environmental influences, proceeding within spatial and temporal domains and constraints (Newnham, 2001). In particular, glucocorticoids alter the trajectory of organ maturation, most notably accelerating late development of the lung and apparently slowing or accelerating aspects of the brain development (Slotkin et al.,1992; Seckl et al., 2000).

Several studies in young animals and adults have demonstrated that stress and increased glucocorticoids can have a major impact on the hippocampal structure (for review see De Kloet et al., 1998; Takahashi, 1998). Animal studies have demonstrated that glucocorticoids interfere with hippocampal normal rates of cell birth and death that occur during development (Gould et al., 1991). The mechanisms by which glucocorticoids induce damage to the hippocampus are not well understood. It is generally accepted that glucocorticoids arrest proliferation and promote differentiation (De Kloet et al., 1988, 1998).

The mapping of glucocorticoid receptors in the rat central nervous system has demonstrated their widespread presence in a large number of nerve and glial cell populations, and also outside the classical stress regions, suggesting other effects not associated with the limbic system (Fuxe et al., 1996).

In the cerebellum, it has been shown that acute prenatal corticosterone treatment decreases cerebellar external granule cell layer width, early in the postnatal development (Velázquez and Romano, 1987), suggesting that this hormone forwards the occurrence of cerebellar granule cell migration. This experimental condition then provides a unique opportunity to evaluate the effects of accelerated development on neuronal structure, since it

is known that the granule-Purkinje cell interactions determine several features of the circuitry established between these two cell types (Altman, 1972a,b).

During brain development, it is important to recognize changes that represent potential early damage caused by the prenatal glucocorticoid exposure, which can produce medium and long-term later consequences. The aim of the present study was to examine the influences of prenatal corticosterone exposure on granule cells migration, Purkinje cell number, Purkinje morphometry (somatic area, nuclear area and dendritic arbor length and width) and ultrastructure in early-aged rats.

Materials and methods

Four control and eight experimental virgin Wistar rats were caged with male rats overnight. The next day, after corroborating the presence of spermatozoa in vaginal smears (gestational day 0), pregnant females were caged individually in temperature and light controlled rooms, and given free access to food and water. Corticosterone was administered (0.2 µg/g of body weight) dissolved in a vehicle containing 2.5% ethanol and 5% propilenglycol in water (Velázquez and Romano, 1987; De la Rosa, 1993; Rugerio et al., 1999). The experimental pregnant rats were injected intraperitonealy with daily, single doses of corticosterone + vehicle, between gestation days 17 to 19. Control rats received only sham injections. At birth, all control and experimental litters were adjusted to six pups each. Neonate rats were sacrificed at postnatal day 12 (PN12), and their cerebella processed for aniline or silver impregnation techniques. The dates for administering corticosterone treatment and for sacrificing the animals were chosen to make comparable the present observations with previous data (Velázquez and Romano, 1987; De la Rosa, 1993; Rugerio et al., 1999). All animal procedures were designed in accordance with local animal rights protection laws and approved by animal rights local committees of the National University of Mexico.

Light microscopy

Eight control and sixteen experimental cerebella of each animal group were fixed in 10% formaldehyde, embedded in paraffin, serially sectioned (7 µm), and stained with Klüver-Barrera technique. Klüver-Barrera stained PN12 cerebella were used to determine Purkinje cell density. Cells were counted in five sections (7 µm thick), and sampled every 35 µm throughout the lobe II (*centralis* ventral lobe). All the Purkinje neurons counted had clearly visible cytoplasm, nucleus and nucleolus.

Sixteen control and thirty two experimental cerebella were processed with the modified chromoargentic technique of Golgi-Kopsch, a silver impregnation previously described (De la Rosa, 1993; Rugerio et al., 1999). Blocks (5 mm^3) from the cerebellar vermis were immersion-fixed in a potassium dicromate-

glutaraldehyde solution (90 mL of 2.22% potassium dichromate solution and 10 mL of 25% glutaraldehyde) for 8 days, washed thoroughly in distilled water, and impregnated in 0.75% silver nitrate for 6 days. Half of this impregnated material (8 control and 16 experimental cerebella) was included in paraffin and serial parasagittal sections (60 µm) were mounted, deparaffinized, cleared, and coverslipped with synthetic resin (Sigma, USA).

Golgi-impregnated material was used to evaluate the effects of prenatal glucocorticoid treatment on granule cell number and migration. Granule cerebellar neurons in different stages of development (Ramón y Cajal, 1981; Nowakowski and Hayes, 2005) were counted in the external granule, molecular and internal granule layers of the lobe II (*centralis* ventral lobe), and referred to a 1 mm^2 area.

Changes in Purkinje neuron dendrites morphology were also evaluated by measuring dendritic length and width occupied by individual dendritic arbors (Patrick and Anderson, 2000). A total of 100 control and 100 experimental cells were evaluated. Only neurons, whose soma (pericaryon) and cell processes appeared fully impregnated, were included in this analysis.

Electron microscopy

The rest of the impregnated cerebella (8 control and 16 experimental) containing the lobe II (*centralis* ventral lobe), were later processed with transmission electron microscopic technique, obtaining semithin and ultrathin sections (Braak and Braak, 1982).

The nucleus and the pericaryon areas of the control (n=100) and experimental (n=100) corticosterone treated Purkinje cells were digitalized in toluidine blue stained semithin sections, which contained the nucleus and nucleolus.

An ultrastructural analysis of Purkinje cell mitochondria, rough and smooth endoplasmic reticulum (RER and SER), Golgi apparatus and ribosomes was realized in ultrathin sections using x3000 amplifications, both in control and experimental groups.

A morphometric analysis of the Purkinje control (n=25) and corticosterone treated (n=50) neurons included the measurement of the perimeter of mitochondria, of RER cistern, and the density of ribosomes in the cytoplasmic area, adjacent to the nucleus and nucleolus in ultrathin sections. The results were expressed in a 10 µm^2 area.

At the ultrastructural level, the number of cells with Cajal (coiled) bodies was counted in 25 control and 50 experimental Purkinje cells. This result was presented in percentages for comparison purposes. In the present analysis realized in ultrathin sections, the presence of one, two or three Cajal bodies, located as a perinucleolar rosette (perinucleolar ring), were considered as indicative of rosette formation.

Statistics

A t-test was applied to almost all the results using

the SAS Program (Statistical Analysis System). In the cases of different phases of development of the granule cerebellar neurons, the length, width of the dendritic arbor, and the pericaryon area of Purkinje cells, a Mann-Whitney test was used (p≤0.01).

Results

The number of Purkinje neurons was estimated across lobe II (*centralis* ventral lobe) sections stained with Klüver-Barrera. No differences in the average
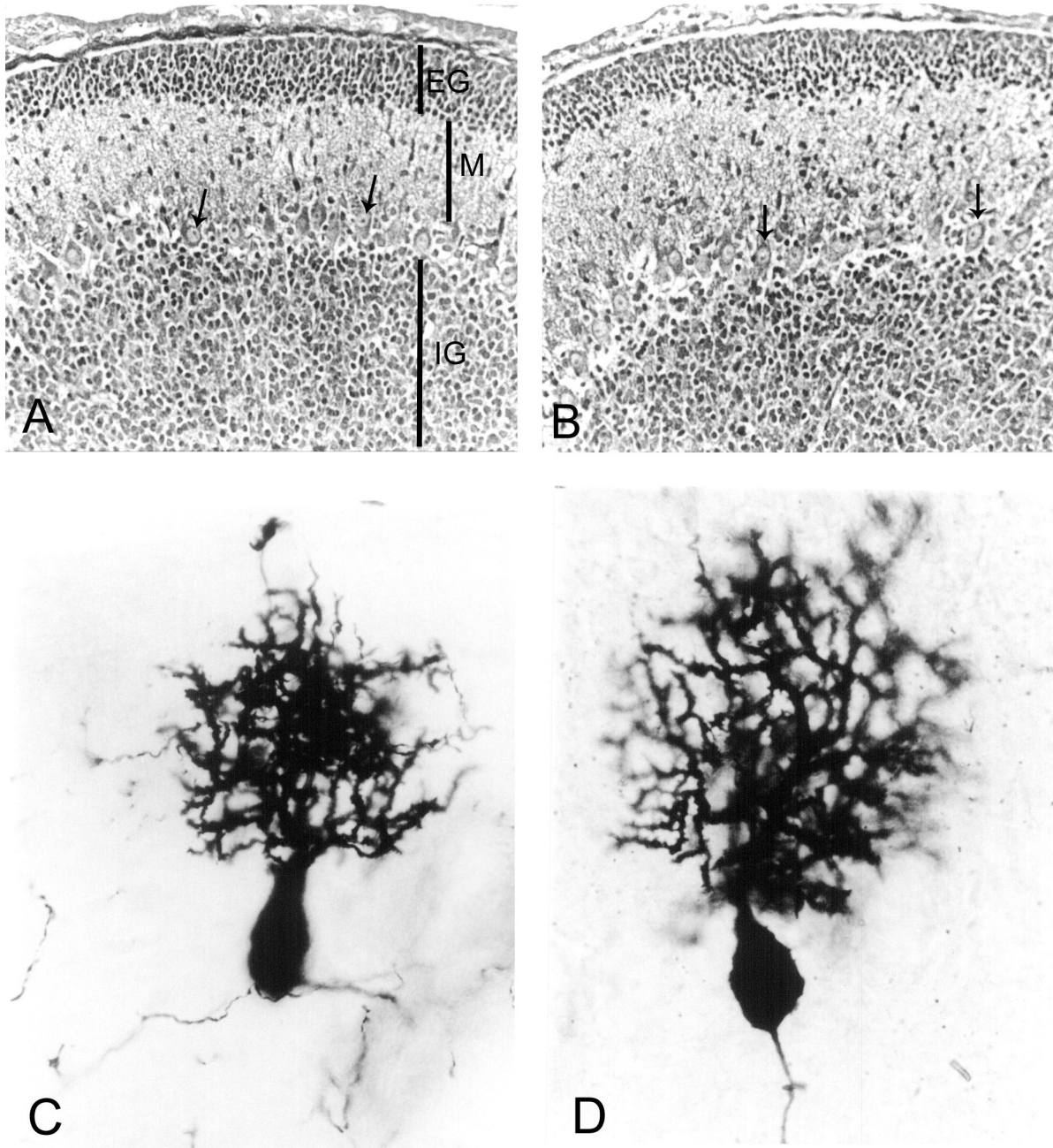
Fig. 1. Effect of the corticosterone prenatal treatment in cerebellum. Light microscopy. Photomicrographs through the cerebellar *centralis* ventral lobe in Klüver-Barrera stained sections (**A:** Control; **B:** Corticosterone treated group). No differences in the number of Purkinje neurons were documented. The different thickness of the cerebellar layers, suggest the forwarding in the migration of the granule cells in the treated group. Arrows: Purkinje neurons. EG: External granule layer. M: Molecular layer. IG: Internal granule layer. x 200. Photomicrographs showing representative Purkinje neurons in control (**C**) and corticosterone treated (**D**) rats. Golgi-Kopsch modified technique. Both length and width of the dendritic arbors are increased in corticosterone treated rats. x 500
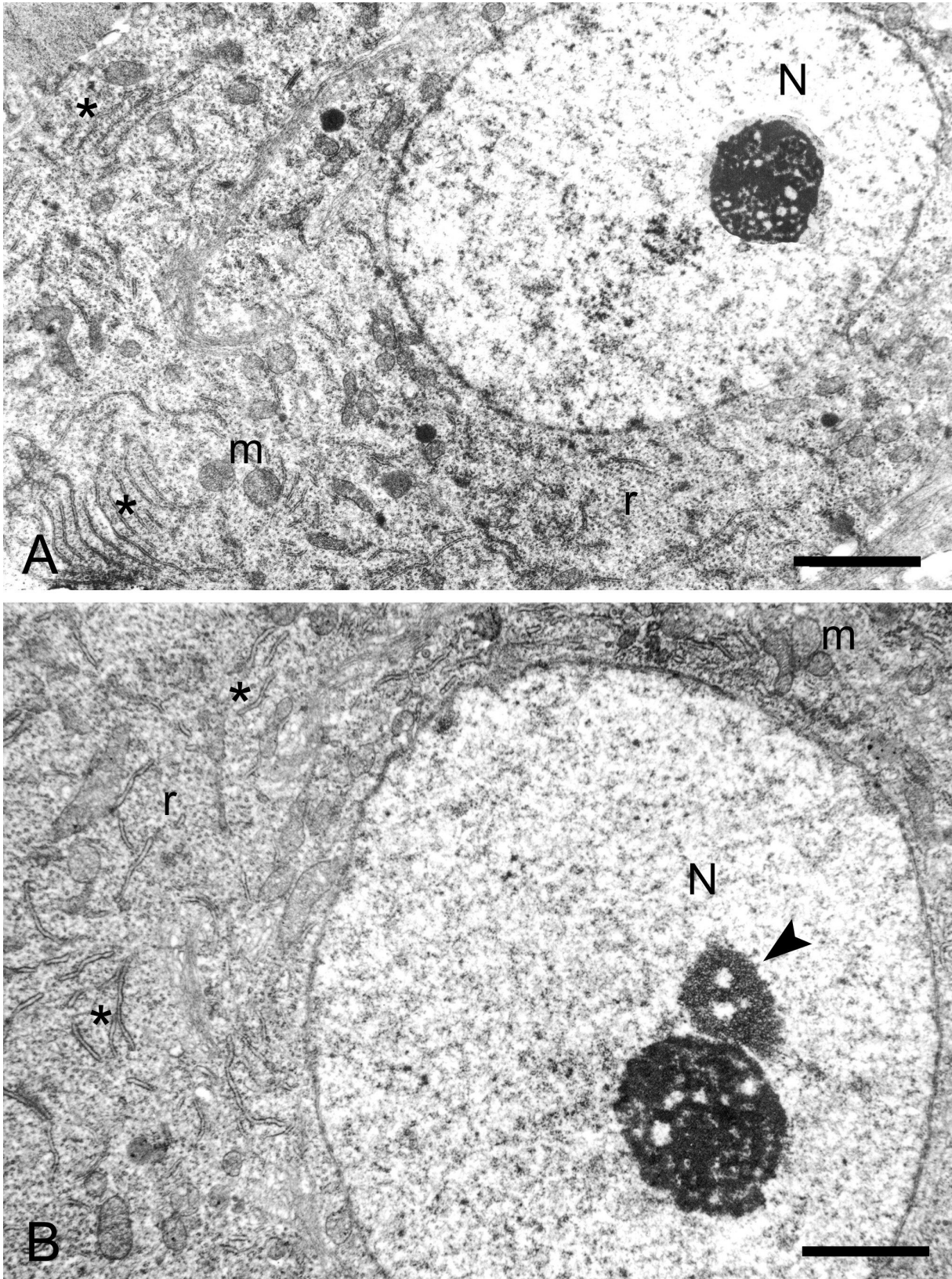
Fig. 2. Effect of the corticosterone prenatal treatment in cerebellum. Electron transmission microscopy. Representative electron micrographs in PN12 control **(A)** and corticosterone treated **(B)** rats. Mitochondria (m), ribosomes (r), nucleus (N). The Nissl bodies (*) have short and less organized cisternae in corticosterone treated animals. The Cajal bodies (arrowhead) are located around the nucleolus in most Purkinje cells in corticosterone treated animals. Note the larger nucleus in **B**. Ultrathin sections. Scale bar: 2.0 μm.

number of Purkinje neurons per section were found among control and experimental rats (74.98±4.63, n=8, and 75.06±2.36, n=16, respectively; mean ± S.E.M.) (Fig 1A,B).

In the Golgi-Kopsch impregnated material, no significant differences were found in the number of granule neurons counted throughout the cerebellar lobe II when control and experimental rats of PN12 were compared (93.47±22.75, n=8, and 75.60±14.50, n=16; mean±S.E.M., respectively). Despite this fact, in the corticosterone treated group the external granule layer showed a 15% decrease in the number of granule cells, while the molecular and internal granule layers displayed 11% and 4% increases respectively in the number of granule neurons. The analysis of the twelve different stages of development of the granule neurons only showed differences in the stage 1, called germ cells (Ramón y Cajal, 1981), in the external granule layer of the experimental group (control median 27.70, n=8; experimental median 0.69, n=16). In the molecular layer there were more migrant forms. Overall, these results suggest that corticosterone treatment forwards the beginning of granule cell migration in corticosterone treated rats. This fact is also evidenced in the Klüver-Barrera stained material, with differences in the thickness of the cerebellar layers in the treated group, with respect to the control (Fig. 1A,B).

The effect of prenatal corticosterone treatment on the Purkinje dendritic arbor morphometry was evaluated by measuring the dendritic length and width in Golgi stained sections. Corticosterone treated animals showed longer dendrites distributed over a greater area extent when compared to control animals (Length median 75.00 vs 62.50. Width median 80.00 vs 75.00; n=100, respectively). (Fig. 1C,D).

The area occupied by the pericaryon in Purkinje neurons showed no differences between either group studied (Control group median: 197.40 μm^2, n=100; corticosterone group median: 182.40 μm^2, n=100). The nucleus of the corticosterone treated Purkinje neurons occupied a statistically significant bigger area than the control group (107.80±3.84 μm^2, n=100, vs. 94.37±3.19 μm^2, n=100; mean ± S.E.M). This difference is evident in the semithin and ultrathin sections (Fig. 2A,B).

In the corticosterone treated ultrastructurally-analyzed Purkinje neurons, the Nissl bodies were less organized and formed by short and few cisternae, while the control group exhibited more organized Nissl bodies, with long cisternae (Fig. 2A,B). However, the perimeter of RER showed no statistically significant differences between either group studied (Control: 2.76±0.32 μm, n=25, and experimental: 3.41±0.28 μm, n=50; mean ± S.E.M.). The mitochondrial perimeter (Control: 8.07±0.84 μm, n=25, and experimental: 8.51±0.65 μm, n=50; mean±S.E.M.) and the free ribosomes density (Control: 624.14±98.41, n=25, and experimental: 770.96 ± 121.69, n=50; mean±S.E.M.) did not show significant differences in the cytoplasmic area analyzed (10 μm^2). The other major organelles in the Purkinje cells, like SER and Golgi apparatus did not show qualitative differences between the groups studied.

When comparing the ultrastructure of the Purkinje cell nucleus, we found that 60% of the prenatally corticosterone treated rats had a perinucleolar rosette, whereas only 40% of the control Purkinje cells presented it (Fig. 2B). It has been proposed that the perinucleolar rosette contains splicing factors. The rosette is considered a diagnostic marker of the end of neuronal differentiation, both *in vivo* and *in vitro* (Santama et al., 1996).

Discussion

In 1996, Fuxe et al. suggested that with increasing age, the endangering actions of glucocorticoids on nerve cells prevail over the neurotrophic ones. Slotkin et al. (1992) suggested that in rats, dexamethasone on gestational days 17, 18 and 19 has specific promotional effects on the development of central cathecholaminergic activity, and can lead to lasting functional abnormalities. The present work shows that the acute prenatal corticosterone administration after the period of Purkinje neuron generation enhances its dendritic arbor, increases the nuclear area, accelerates perinucleolar rosette appearance and decreases the development of the Nissl bodies soon after birth (PN12). We think that these changes could represent a transient condition (a plastic adaptation) or could produce a medium or long-term later consequences. Supporting the former possibility, in the same model, studies at PN20 have demonstrated a diminished dendritic growth in the Purkinje cell (Rugerio et al., 2003).

Our observations that Purkinje neurons have both increased dendritic length and width in 12 days old corticosterone treated rats, contrast with previous works documenting detrimental effects of steroids on Purkinje cells dendritic development. In these studies, however, steroids were administered continuously all along postnatal development and their effects were analyzed in the adult stage (Bohn and Lauder, 1978). We instead, injected pregnant rats with corticosterone only three days during prenatal development, soon after the period of Purkinje neurons generation. This technical discrepancy might in part explain why Purkinje neurons showed a different response in our experimental series. It might be that Purkinje neurons display different structural responses to steroids depending upon the ongoing developmental stage, the duration of the treatment and the impact on the short, medium and long terms.

The outgrowth of dendrites is a complex process that can be modulated through different mechanisms (Nowakowski and Hayes, 2005). An intrinsic genetic program may control the first step of dendritic growth (Berry and Bradley, 1976; Nowakowski and Hayes, 2005). Once reached a certain, genetically controlled length, the growth and geometry of dendritic arbors highly depend on trophic interactions established

between dendrites and axons impinging upon them (Sotelo, 1978; Nowakowski and Hayes, 2005; Mc Allister et al., 1995).

With our experimental series we cannot establish if the glucocorticoids treatment can alter the genetically controlled phase of Purkinje neurons dendritic growth. Although a recent study has shown that a single low dose of dexamethasone can cause profound perturbation of nuclear transcription factors in the fetal rat brain (Slotkin et al., 1998), it is known that major events on Purkinje neuron dendritic development depend on trophic interactions between these cells and granule neurons (Altman, 1972a,b, 1976a-c; Berry and Bradley, 1976; Dumesnil-Bousez and Sotelo, 1992). According with Baptista et al. (1994) local epigenetic factors, provided principally by the granule neuron, regulate the proper Purkinje cell differentiation resulting in dendrites with spines receiving synapses. Also, during development, changes in the electrical activity might control dendrite growth pattern of Purkinje neurons (Schilling et al., 1991).

Some previous results (Velázquez and Romano 1987; De la Rosa 1993; Rugerio et al., 1999) and the present study suggest that corticosterone treatment forwards granule neurons migration. In our experimental model the decreased stage 1 granule cells suggest that corticosterone promote differentiation as De Kloet proposed in the hippocampus (De Kloet et al., 1988, 1998). It is possible that an earlier establishment of synaptic contacts between parallel fibers and dendritic spines of Purkinje cells underlie the effect of steroids treatment on Purkinje neurons.

The development of the nervous system proceeds in orderly fashion according to strict developmental timetables. When the progression of these schedules is disrupted, major anatomical and functional alterations are observed in the nervous system (Nowakowski and Hayes, 2005). In general, delaying the progression of development programs leads to increases in neuron size, while reduced neuron size would likely result from accelerated development (for review see Gould, 1977). Our results disagree with the latter statement. In the semithin sections, no differences were found in the pericaryon area between controls and rats prenatally treated with corticosterone.

The larger size of the nucleus in the Purkinje neurons prenatally treated with corticosterone suggest that this effect is due to the hormone treatment and could be related to the mechanism of action of steroid hormones on the nucleus of target cells to stimulate genetic transcription (Slotkin et al., 1998). Due to the fact of the important reorganization of cytoplasmic and nuclear components during development (Spector, 1993), we analyzed at the ultrastructural level the major Purkinje cell organelles. Also, a structure considered to be a key factor during neuronal maturation: the Cajal body. This structure, which has a high affinity for silver, was first observed by Ramón y Cajal in 1903 (Nowakowski and Hayes, 2005) and was named by him

as the "accessory nucleolar body". The reorganization of these structures and their perinucleolar location (rosette) agrees with a marked increase in transcriptional activity present during late stages of neuronal differentiation, of some neuronal types, such as hippocampus pyramidal neurons (Santama et al., 1996), Purkinje (Dunn et al., 1998) and granule neurons (Lafarga et al., 1997) of the cerebellum. The Cajal bodies are absent in proliferative cells (Vázquez et al., 2003) and in apoptotic neurons (Lafarga et al., 1997).

In cultured Purkinje cells of mouse, the perinucleolar rosette appears during the third postnatal week (Dunn et al., 1998). Our results suggest that a larger number of PN12 Purkinje neurons in the experimental group might be at a higher transcriptional activity state. With this model, this aspect of the cellular differentiation of Purkinje cells was favored.

Plentiful information on the application of glucocorticoids during development suggest a progress in the process of neuronal differentiation (Hall, 1982; De Kloet et al., 1988) and also the development of neuron membrane properties (Romano et al., 1985; Fuentes-Pardo et al., 1990).

On the other hand the poor development and lack of organization of the Nissl bodies in the corticosterone impregnated Purkinje neurons suggest that other aspects of the cellular differentiation were delayed.

The current model of corticosterone-induced morphological changes in Purkinje neurons of developing rats, offers an interesting possibility to study and understand the plastic changes that result when the progression of normal programs of development is altered.

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