Histol Histopathol (2021) 36: 195-205

http://www.hh.um.es

Histology and Histopathology
From Cell Biology to Tissue Engineering

ORIGINAL ARTICLE **Open Access**

Argentic staining reveals changes in cerebellar tissue organisation by prenatal glucocorticoid administration in rats

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Summary. It was almost 150 years ago that Golgi revolutionised histology with silver-based stains. Major advances in knowledge of the nervous system became possible because of silver impregnations. Silver staining combined with classical histological staining, cytochemistry methods, and electron microscopy is useful for studying mechanisms and components at subcellular, cellular, and tissue levels. Despite the advantages of silver staining, its use has decreased over time. The aim of this work was to use argentic staining to study the cerebellar effects of controversial prenatal glucocorticoid (GC) therapy. At postnatal day 12 (P12), the cerebellum of corticosterone (CC)-treated rats impregnated with AgNOR staining exhibited diminished thickness of the external granule layer (EGL) and irregular Purkinje cell arrangement. There was a greater number of nucleoli and nucleolar organiser regions (NORs) in 24% of Purkinje cells. Cerebellar granule neuron progenitor (CGNP) cells of the EGL showed a decrease in cellular density (confirmed by proliferating cell nuclear antigen [PCNA] immunolocalization) and NORs. At postnatal day 6 (P6), the Golgi-Kopsch technique allowed us to observe disturbances in the distribution pattern of CGNP cells (during proliferation, migration, and differentiation) and premature growth of the Bergmann glia. Our findings reveal disturbances in the cerebellar development program with early cellular and tissue changes.

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DOI: 10.14670/HH-18-291

Key words: Corticosterone, Preterm delivery, Developmental biology, Silver staining, Nucleolar organiser region

Introduction

Argentic stains have been a very valuable tool for nervous system studies and neuronal morphology recognition (DeFelipe, 2006; Agnati et al., 2007; Azmitia, 2007). These allow for visualisation of the morphological details of arborisation, dendritic spines, and glia (Czechowska et al., 2019; Du, 2019). Moreover, argentic staining is a bioanalytical tool (Newman and Jasani, 1998), which, when combined with classical histological staining, cytochemistry methods (Torres-Montaner et al., 2000; Kasai et al., 2004; Vints et al., 2019), and electron microscopy (Gancevici and Petrovici, 1993; Larriva-Sahd, 2004), can expose mechanisms and elements at subcellular, cellular, and tissue levels (Escalona et al., 2002; Rivas Manzano et al., 2010; Goldman et al., 2016).

The Golgi staining technique was developed in the 19th century and is still in force (Torres-Fernandez, 2006; Czechowska et al., 2019; Vints et al., 2019). This technique is based on impregnation with silver chromate, the product of the reaction between potassium dichromate and silver nitrate (Spaceck, 1992; Grizzle, 1996). The use of silver staining is not restricted to the study of the nervous system. Silver staining allows the study of various structures, such as reticular fibres, melanin, fungi, karyotype, chromosome banding, calcium depots, and nucleolar organiser regions (NORs) (Grocott, 1955; Humason, 1967; Rufas et al., 1983; Kavalco and Pazza, 2004; Kiernan, 2008).

AgNOR staining described by Ploton et al. (1986) involves impregnation of proteins associated with NORs

(Derenzini, 2000). The affinity of silver salts to nucleolar proteins, nucleolin, and nucleophosmin (Hernandez-Verdum, 1983, 2006; Roussel and Hernandez-Verdum, 1994) makes this technique useful for the analysis of nucleolar dynamics during cell cycle (Sirri et al., 2000), metaphase (Grizzle, 1996), meiotic prophase (Villalobos et al., 2019), spermatogenesis (Hofgärtner et al., 1979) and nucleologenesis (Ochs et al., 1985). Additionally, AgNOR distribution has allowed for the study of metabolic activity, particularly in cancer cells (Crocker and Nar, 1987; Slowiska-Klencka et al., 2004; Treré et al., 2004; Smetana et al., 2005; Stemberger-Papić et al., 2006; Monti-Hughes et al., 2020) and apoptotic cells (Torres-Montaner et al., 2000; Kito et al., 2005). In the same way, this method allows for the evaluation of the nucleolar transcriptional activity of ribosome formation during the interphase because AgNOR stains the fibrillar centre (Roussel et al., 1996). For this reason, this staining has allowed for the evaluation of neural activity (González-Pardo et al., 1994; Garcia Moreno et al., 1997).

Glucocorticoid (GC) administration in pregnant women with the risk of preterm delivery to prevent respiratory neonatal complications can trigger adverse developmental side effects. GC therapy in animal models and clinical trials have shown neuro-developmental abnormalities and permanent neuromotor and cognitive deficits (Yeh et al., 2004; Lucassen et al., 2009; Tijsseling et al., 2012). In addition to negative effects on brain development, other organs are also affected (Seckl, 1998; Matthews, 2000). Previously, we described that in rats, prenatal corticosterone (CC) administration has been shown to promote neuromotor behavioural alterations in early reflexes (Rugerio-Vargas et al., 2003), and morphological changes in Purkinje cells, including higher growth of dendritic arbours (Rugerio-Vargas et al., 1999), increased nuclear areas, early emergency of perinuclear Cajal bodies, and decreased Nissl bodies (Rugerio-Vargas et al., 2007).

Animal models have shown regulatory effects of GC on neuronal growth and differentiation (De Kloet et al., 1998; Seckl, 1998). GC decreases cerebellar DNA content (Velázquez and Romano, 1987), increases neuronal death by oxidative damage (Ahlbom et al., 2000), and produces cerebellar hypoplasia (Limperopoulos et al., 2005; Volpe, 2009). According to Noguchi et al. (2008), GC administration to mice at postnatal day 4 (P4) and 10 (P10) produced apoptosis of cerebellar granule neuron progenitor (CGNP) cells at the external granule layer (EGL) and a permanent decrease in neurons in the internal granule layer (IGL). Moreover, GC therapy in newborns can disturb cerebellar maturation (Haldipur et al., 2011; Tam et al., 2011). Despite the adverse effects, GC administration is the standard treatment for pregnant women with the risk of preterm delivery and it is the standard treatment for preterm neonates. Using argentic techniques in combination with cytochemistry in an established prenatal rat model of CC therapy, this work tested the early changes in cerebellar cortex architecture.

Materials and methods

Animals

Wistar rats were housed in individual caging with *ad libitum* access to water and standard chow. All experimental studies were conducted in accordance with the Animal Studies Committee of the National Autonomous University of Mexico (UNAM for its initials in Spanish) and the Mexican National Protection Laws of Animal Welfare NOM-062-ZOO-1999, for technical specifications for production, care and use of laboratory animals.

Pregnant rats (n=8) were divided into an experimental group (n=4) and a control group (n=4). The experimental group received intraperitoneal injections of CC (Sigma) (0.2 μg/g body weight/2.5% ethanol and 5% propylene glycol in 0.5 mL volume) on gestational days 17 (G17), 18 (G18) and 19 (G19). Corticosterone was used to ensure efficient placental penetration and high gestational exposure of GC because corticosterone is the rodent endogenous glucocorticoid. The control group was injected with the vehicle only. The rat cerebellum on postnatal day 0 is similar to the third trimester of human gestation or premature delivery (Biran et al., 2012; Sathyanesan et al., 2019). Therefore, the glucocorticoid was administered in G17-19, when, in terms of cellular proliferation, they show similarities with the human cerebellum during the second trimester of gestation. Experiments were done on newborn (P0), postnatal day 6 (P6) and postnatal day 12 (P12) rats.

AgNOR method (Ploton et al., 1986)

To impregnate NORs, the cerebellums of P12 offspring were fixed in 10% formalin, dehydrated in gradual alcohol, and embedded in paraffin. Vermis medial para-sagittal sections (5 μm) were deparaffinised and rehydrated. Tissue slides were incubated in 33% silver nitrate (7761-88-8, Merck) dissolved in a solution of 1% gelatine (H219-59, Baker) and 0.5% formic acid (0128, Baker) freshly prepared for 45 minutes at room temperature in darkness. Tissue slides were generously rinsed with distilled water, dehydrated, cleared, and mounted.

The cell density of CGNP cells and the thickness of the EGL were evaluated with a microscope micrometer rule at 40x. Ten sections, one every 25 μm of the anterior lobe were analysed. The numbers of nucleoli and NORs were counted in CGNP and Purkinje cells. Also, the nuclear area, NOR area and their ratio were measured using the axiovision software, free version from Zeiss. The measurement method involved defining the outer edge of the nuclear envelope. The program automatically generates an excel spreadsheet with the measurements.

Proliferating cell nuclear antigen (PCNA) immunohistochemistry

The cerebellums of P12 rats were fixed in 4% paraformaldehyde, dehydrated in graded alcohol, and embedded in paraffin. Vermis medial para-sagittal sections (5 μm) were deparaffinised, rehydrated, and rinsed with Phosphate-Buffered Saline (PBS) for 20 minutes. Tissue slides were incubated with a 3% H_2O_2 blocking solution for 40 minutes and washed with water. Antigen retrieval was carried out with citrate buffer (pH 6) (HK86E4, BioGenex). Sections were incubated with universal blocking buffer for 10 minutes and with normal goat serum blocking solution for 30 minutes. Monoclonal primary antibody to the proliferating cell nuclear antigen (anti-PCNA) (N1529, DAKO) (1:100) was incubated for 60 minutes. The second antibody goat anti-Mouse polyvalent IgG biotin conjugate (Super Sensitive MultiLink, HK340-9K, BioGenex) was incubated for 20 minutes after washing with PBS. Slides were incubated with Peroxidase Conjugated Streptavidin Super Sensitive Horseradish Peroxidase (HRP) (HK330-5K, BioGenex) for 20 minutes and washed with PBS. The signal was visualised with the chromogenic substrate 3-amino-9-ethylcarbazole (HK092, BioGenex). Finally, slides were counterstained with Gill's haematoxylin and mounted with Cytomation Faramount Aqueous mounting medium (S3025, DAKO). Ten sections, one every 25 μm of the anterior lobe were analysed.

Golgi-Kopsch method

The cerebellums of P0, P6 and P12 animals (n=4) were incubated with fixative Golgi-Kopsch (glutaraldehyde-dichromate) in darkness for 8 days at room temperature. The cerebellum was rinsed with distilled water and impregnated with 0.75% aqueous solution of silver nitrate (7761-88-8, Merck) in darkness for six days. Cerebellum was dehydrated with gradual alcohols after washing with distilled water and was embedded in paraffin. Sections that were 60 μm in size were deparaffinised and mounted with synthetic resin (44581, Sigma). The area occupied by the CGNP and CGNP-derived migratory cells in the anterior lobe from 10 serial sections was measured according to the 10 stages described by Ramón y Cajal (1909-1911) (for review see Nowakowski and Hayes, 2005). The morphometry of Bergmann glia was evaluated by microscope micrometer rule at 40x by measuring the major axis of the soma and the length and number of processes occurring in the cell body.

Statistical analysis

The results are expressed as means ± the standard deviation (STDEV). The equality of variances was evaluated with the F-test. Comparisons between control and corticosteroid groups were made with the Student's T-test using Prism 6.0 software (GraphPad, San Diego, California, USA). Results are presented as the mean ± STDEV. The significance was defined as $p<0.05$. Some numerical data with statistical differences were expressed in percentages. Statistical analysis is included in Supplementary material 1-3.

Results

AgNOR impregnation in the cerebellar cortex

To determine the AgNOR that would be useful to evaluate disturbances in cerebellum development, we analysed the morphology and tissue organisation in impregnated sections of the control cerebellum. In the rat P0 (corresponding to the late second trimester or beginning of third trimester in humans), a second wave

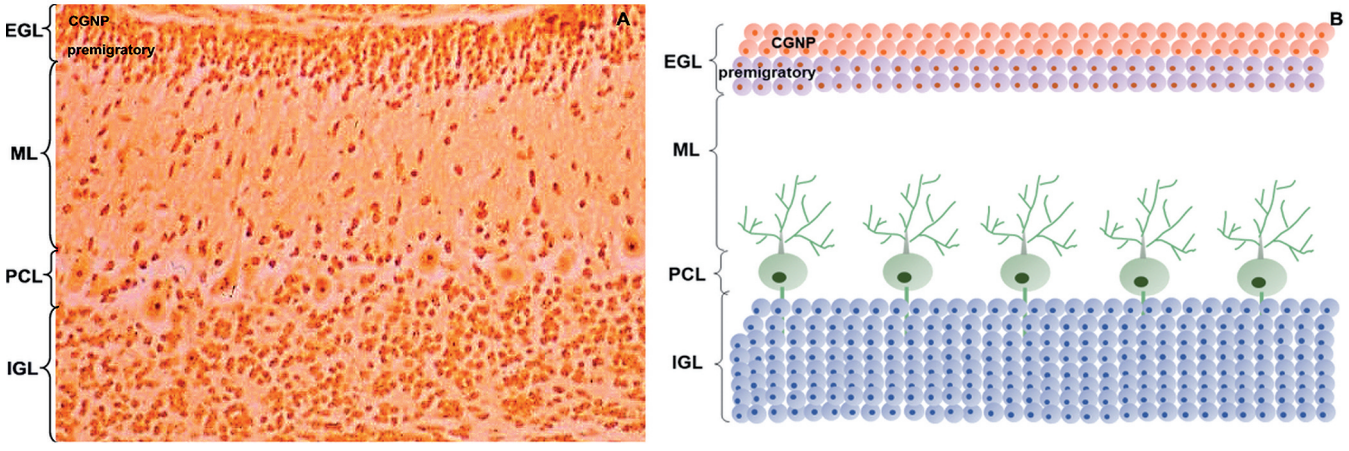

Fig. 1. Cerebellar cortex stained with AgNOR method. P12 cerebellar cortex is in development and presents the EGL, ML, PCL, and IGL. The EGL is formed, in turn, by a peripheral proliferative layer (CGNP) and a premigratory layer **(A)**. Schema of cerebellar layers **(B)**. EGL, external granule layer; ML, molecular layer; PCL, Purkinje cell layer; IGL, internal granule layer; CGNP, cerebellar granule neuron progenitor; P12, postnatal day 12.

of granule cell proliferation occurs, and their migration begins. Cells of the EGL migrate to the IGL through the molecular layer (ML) during the following 21 days, resulting in a transition in the cerebellum from five immature layers to three mature layers. Since the rat cerebellum is in development at P12, the AgNOR method allowed us to stain the initial five layers (Fig. 1). Therefore, we proceeded to evaluate the effect on the cerebellum of CC injection during rat pregnancy.

AgNOR impregnation reveals differences in external granule layer (EGL) thickness

The cerebellums of experimental P12 rats showed a decrease in EGL thickness. According to the measurements, the EGL thickness decreased from 77.94±9.64 to 37.83±5.67 μm in the CC group compared to the control ($p<0.001$) (Fig. 2). Moreover, the cell density in the EGL was reduced by the CC effect (8.35±0.865 cells/1000 μm^2 vs 7.11±0.558 cells/1000 μm^2).

Proliferation and protein synthesis in the EGL

Haematoxylin eosin staining is the most useful stain for evaluating general histological tissue structure and characteristics. In this way, AgNOR impregnation is a better method than haematoxylin eosin staining because, according to Cresta and Alves (2007), AgNOR impregnation also correlates with cellular proliferation and protein synthesis levels.

The EGL is formed by an outer region of the EGL, located close to the pial surface, which corresponds to the dense proliferating precursor cell layer with the CGNP. The inner region of EGL corresponds to less densely packed post-mitotic premigratory neurons. We found reductions in the nuclear and NOR area in CGNP cells from the CC group (Fig. 3A-B). The size of the NORs and nuclear area in the control was 0.74±0.4 μm^2 and 16.75±4.18 μm^2, respectively. In the P12 cerebellum of the experimental group both parameters decreased; the NOR area diminished to 0.62±0.3 μm^2 ($p<0.05$) and the nuclear area was 12.6±3.5 μm^2 ($p<0.001$) (Fig. 3C-D). There was no relationship between the mean area of the NOR and the nuclear area of the control and CC group (0.05±0.03 μm^2 vs 0.049±0.02 μm^2).

Corticosterone (CC) decreases EGL proliferation

AgNOR impregnation revealed cellular proliferation in the EGL; however, we immunolocalised the PCNA to correlate with proliferation with a more specific marker. The cerebellums of P12 rats showed a decrease of CGNP positive to PCNA of the peripherical proliferative layer of the EGL from CC group (2.53±0.24 cells/400 μm^2 vs 3.82±0.27 cells/400 μm^2, $p<0.001$) (Fig. 4).

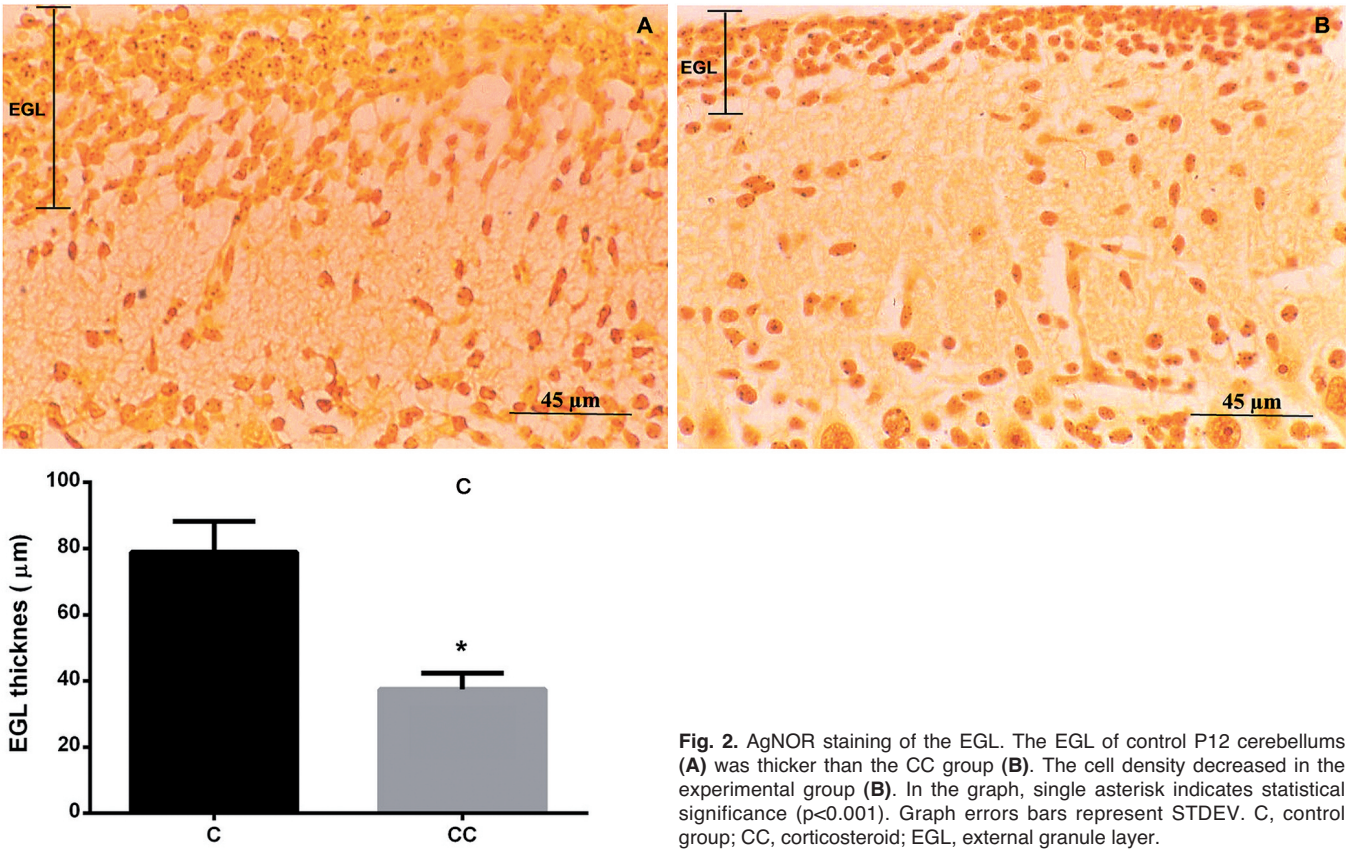

Fig. 2. AgNOR staining of the EGL. The EGL of control P12 cerebellums **(A)** was thicker than the CC group **(B)**. The cell density decreased in the experimental group **(B)**. In the graph, single asterisk indicates statistical significance ($p<0.001$). Graph errors bars represent STDEV. C, control group; CC, corticosteroid; EGL, external granule layer.

Corticosterone (CC) disturbs the Purkinje cell layer (PCL)

In addition to disturbances in the EGL in P12 rats because of CC injection, we also observed modifications in the Purkinje cell layer (PCL). Purkinje cells are born during early development, which gives rise to 2-3 lines. During postnatal life of the rat (P0-P4), Purkinje cell lines move and line up to generate a monolayer with a highly aligned arrangement (Altman, 1972). AgNOR impregnation allowed us to demonstrate irregular arrangement in their Purkinje cell layer (Fig. 5A,B). Nevertheless, the cell density of the PCL was similar in the experimental group and control group (10.08±0.52 cells/10000 μm^2 vs 10.06±0.46 cells/10000 μm^2).

Purkinje cells of the control group have a single

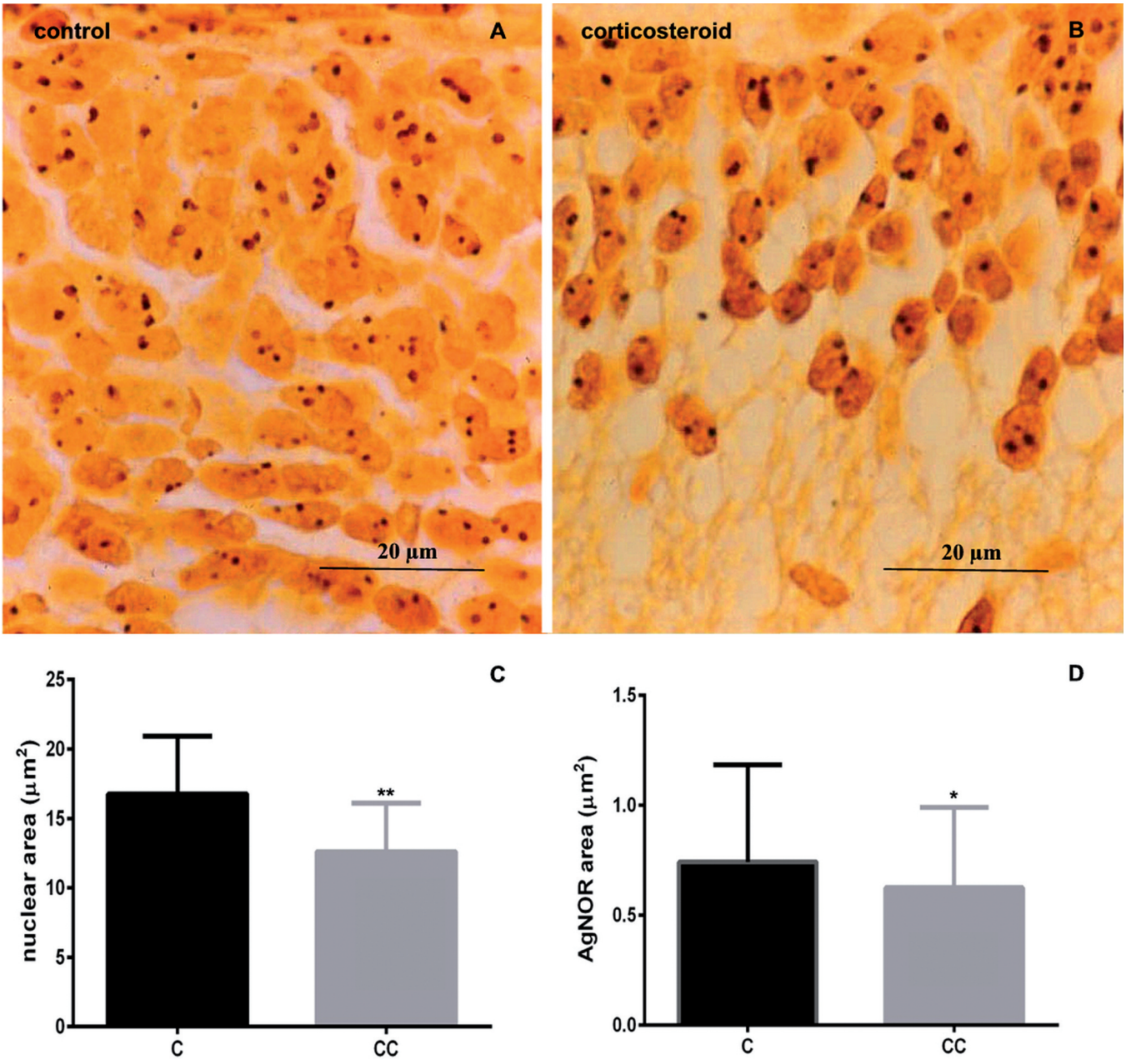

Fig. 3. Proliferation of the EGL. Cellular proliferation and protein synthesis evaluated by AgNOR staining in P12 cerebellums of the CC group showed a decrease in nuclear **(B-C)** and AgNOR **(B-D)** area in the CGNP of the EGL. In the figure, single ($p<0.05$) and double ($p<0.001$) asterisks indicate statistical significance. Graph errors bars represent STDEV. C, control group; CC, corticosteroid; CGNP, cerebellar granule neuron progenitor; EGL, external granule layer.

nucleolus. However, 24% of Purkinje cells in the experimental group exhibited a greater number of nucleoli (2 nucleoli in 13.8%, 3 nucleoli in 7.4% and 4 nucleoli in 2.8% of Purkinje cells) (Fig. 5C,D), especially in Purkinje cells with irregular arrangement. Moreover, the number of NORs per nucleus was higher but statistically non-significant (CC, 2.43±0.92 vs control, 2.25±0.91).

In contrast to the effect seen in the EGL, the PCL showed an increase in NORs and the nuclear area in the prenatal CC group. The NOR and nuclear area in the control was 0.72±0.74 μm^2 and 56.75±11.14 μm^2, respectively. In the P12 cerebellum of the experimental group, the NOR area increased to 1.0±0.97 μm^2 (statistically non-significant) and the nuclear area increased to 72.62±14.9 μm^2 ($p<0.001$) (Fig. 5E,F). However, there was no relationship between the mean area of the NOR and the nuclear area in the control and CC group (0.014±0.013 μm^2 vs 0.02±0.02 μm^2).

Golgi-Kopsch method to show changes in cellular migration and differentiation

We used the Golgi-Kopsch method to evaluate the effect of the decreased CGNP proliferation on cell differentiation. Distribution and cell morphology from the EGL to the IGL were analysed according to the 10 stages described by Ramón y Cajal (1909-1911). The Golgi method is an excellent neuronal morphology marker; unfortunately, the percentage of cells impregnated using the Golgi-Kopsch method is very low (5-10%) and does not reflect their real status. Moreover, a proper statistical analysis cannot be done, and we have therefore only shown the mean.

There were no differences in the migration and differentiation patterns of the anterior lobe of P12 rats

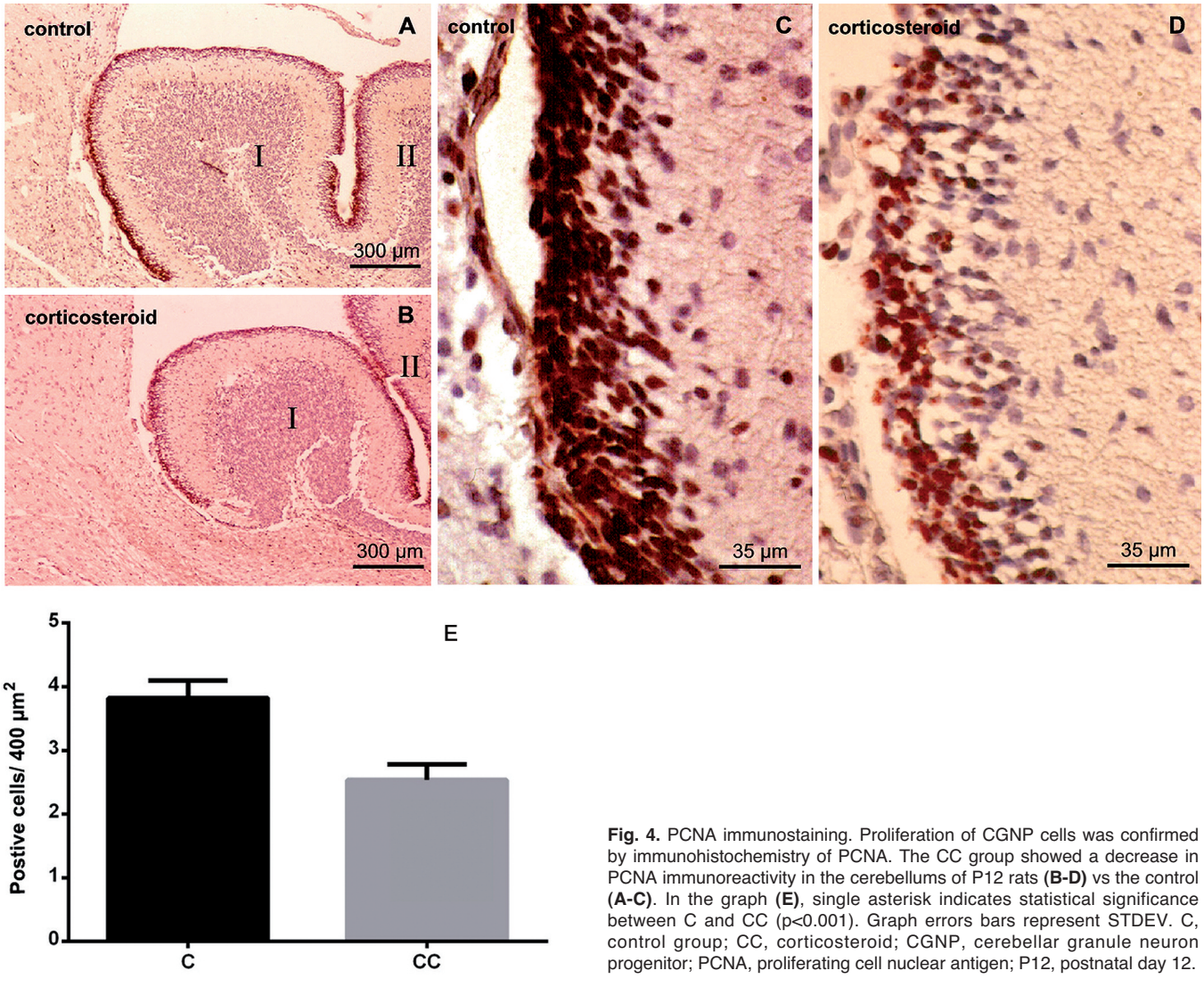

Fig. 4. PCNA immunostaining. Proliferation of CGNP cells was confirmed by immunohistochemistry of PCNA. The CC group showed a decrease in PCNA immunoreactivity in the cerebellums of P12 rats **(B-D)** vs the control **(A-C)**. In the graph **(E)**, single asterisk indicates statistical significance between C and CC ($p<0.001$). Graph errors bars represent STDEV. C, control group; CC, corticosteroid; CGNP, cerebellar granule neuron progenitor; PCNA, proliferating cell nuclear antigen; P12, postnatal day 12.

from the experimental group and the control group. However, P0, P6, and P12 rats of the experimental group exhibited decreases in CGNP lineage when all stages were added, regardless of the layer. It was found that 43% less cells were impregnated in the P0 cerebellum of the CC group (26.9 cells/mm^2 vs 47.1 cells/mm^2), and 20% less cells were impregnated in P6 cerebellums (84.61 cells/mm^2 vs 104.45 cells/mm^2) and 21.5% less cells were impregnated in P12 cerebellums (73.4 vs 93.6/mm^2).

Only P6 rats of the CC group showed changes in CGNP lineage distribution and morphology (Fig. 6).

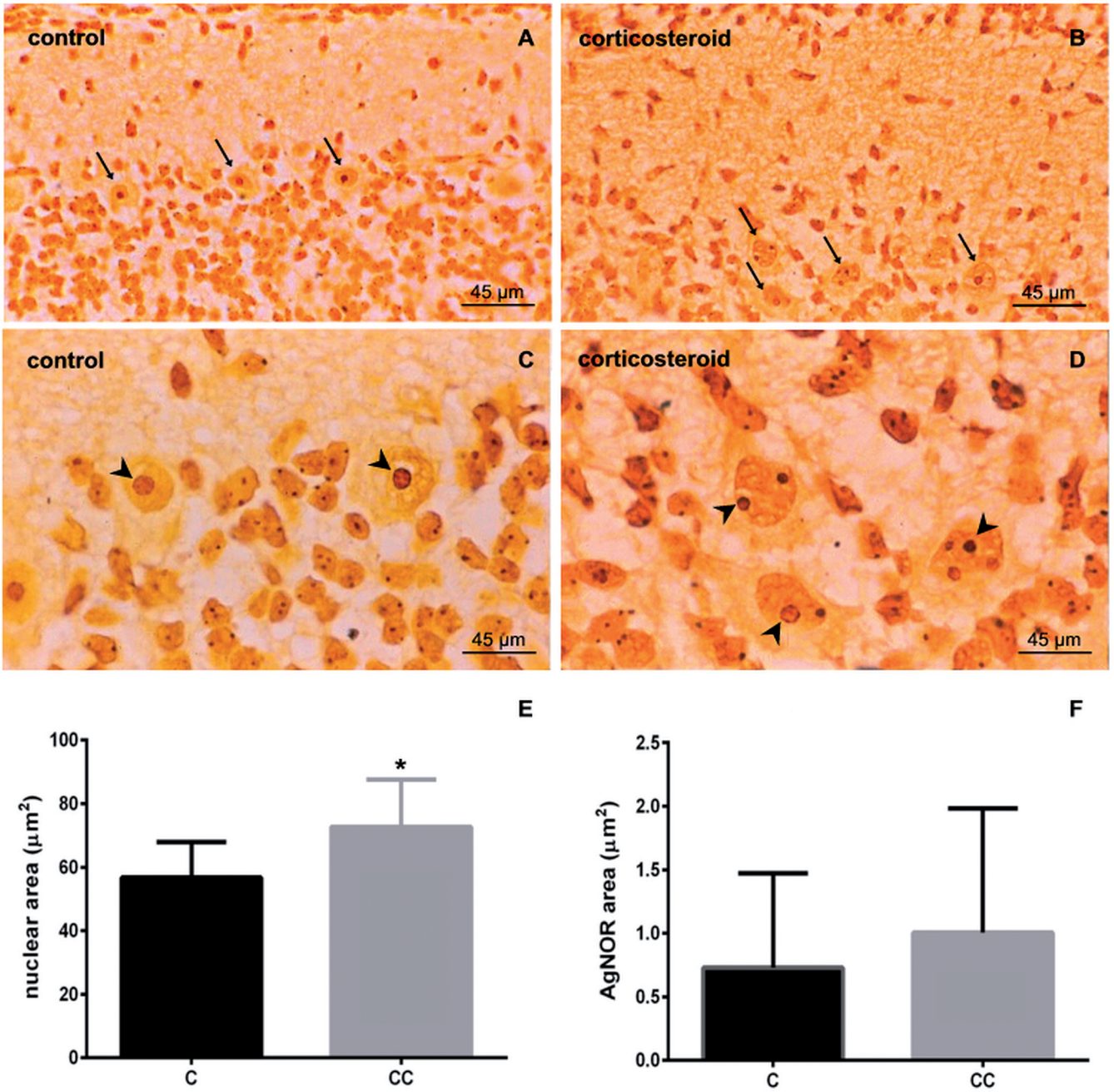

Fig. 5. Corticosteroid effects on Purkinje cells. AgNOR staining in the cerebellar cortex of P12 rats showed PCL (arrow) aligned in the control group **(A)** but disordered in the CC group **(B)**. Purkinje cells of the control group **(C)** showed a single nucleolus (head arrow), but the CC group exhibited a greater number of nucleoli **(D)**. AgNOR and nuclear area values increased in the CC group **(E-F)**. In the graph, single asterisk indicates statistical significance between C and CC ($p<0.05$). Graph errors bars represent STDEV. C, control group; CC, corticosteroid.

According to the a-j stages described by Ramón y Cajal (1909-1911) and Jacobson (1991), the P6 cerebellum exhibited an increase in CGNP staining (a stage; CC, 37.6 cells/mm^2 vs control, 6.2 cells/mm^2) in the peripheral proliferative layer. The CC group showed a decrease in the migratory stages per mm^2 (stages e and h; 8.7 vs 54.4 and 5.5 vs 24.9, respectively) of both the ML and PCL. Moreover, the CC group exhibited an increase in young granule cell stage per mm^2 (stages i and j; 15.3 vs 3.5 and 1.08 vs 0.05, respectively) in the IGL. Furthermore, only the CC group showed fully differentiated mature cells per mm^2 (0.03) (Fig. 6).

Bergmann cells are radial astrocytes with processes that extend to the pia mater favouring the migration of differentiating CGNP lineage from the EGL to form the IGL. For this reason, we analysed the morphometric parameters of Bergmann glia at P0, P6 and P12. The major axis of the Bergmann soma was 10% bigger in the CC group than the control group of P0 rats. The Bergmann glia of the CC group showed 24% more processes at P0 and 16% more processes at P6. However, at P12 the major axis decreased in the experimental group (22% vs 47%), suggesting less growth of the soma. Analysis from P0 to P12 showed that the CC group had 67% more processes and the control group had 128% more processes indicating a decrease in ramification due to CC.

Discussion

It was almost 150 years ago that Golgi developed chromoargentic stains (Pannese, 1999), which initially allowed for great advances in histological knowledge and studies of the nervous system (DeFelipe, 2006; Agnati et al., 2007; Azmitia et al., 2007). In this work, we performed AgNOR and Golgi-Kopsch techniques, which are silver staining methods. AgNOR impregnation is related to proliferation and protein synthesis (Cresta and Alves, 2007) because it allows for the staining of proteins of NORs, which are portions of DNA that code for ribosomal RNA (rRNA).

The Golgi-Kopsch method allowed us to visualise the architecture of the impregnated neuron and the associated morphological characteristics with brain function (Das et al., 2013). Despite the advantages offered by silver staining, its use has decreased over time. Because GC is associated with the development of cardiovascular, metabolic, and neuroendocrine diseases (Seckl, 2004), our aim in this paper was to employ silver staining to evaluate the effect of prenatal CC on early changes in the cerebellar cortex structure of rats.

In this work, through AgNOR staining we observed a decrease in thickness of the EGL because of prenatal administration of CC. This effect was also previously observed by Haldipur et al., (2011) in post-mortem studies of preterm labour infants, suggesting disturbances in cerebellum development by preterm delivery and disturbances in development in an *ex utero* environment.

Furthermore, AgNOR analysis can be quantitative by counting each silver-stained dot per cell method and by the morphometric method, which consists of the area occupied by the silver-stained structures within the nuclear profile (Trerè, 2000). Moreover, AgNOR staining has been employed in both humans and rodents to study disturbances in neuronal transcriptional activity related to aging and disease (Qü et al., 1994; Bielau et al., 2005). Our results showed a decrease in the nuclear area and the NOR area of EGL cells in the CC group, which suggests a drop in both proliferative activity and protein synthesis (Cresta y Alves, 2007). The decrease in proliferation in P12 CC rats was corroborated by PCNA immunohistochemistry. In the same way, dexamethasone administration during the critical prenatal period decreases proliferation of embryonic CGNP cells and accelerates the maturation and occurrence of epigenetic changes in animal models, suggesting that disturbances in intrauterine programming may predispose subjets to

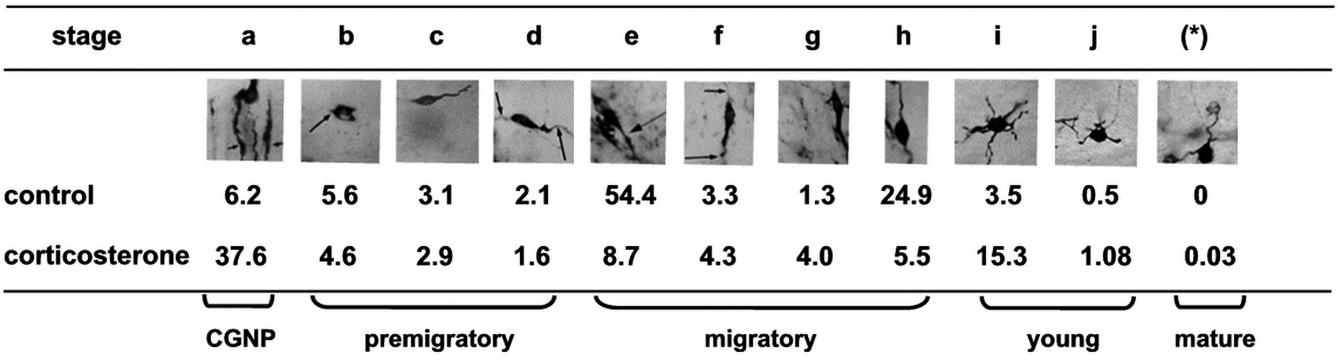

Fig. 6. Morphological stages in the cerebellum. Differences in the morphological stages of the granule cells along to the anterior lobe were seen in P6 rats in the CC group and control group. Stage a corresponds to CGNP cells, stages b-d correspond to premigratory cells, stages e-h correspond to migratory cells, i-j correspond to young granule cell stage and (*) corresponds to mature cells. Values represent the mean of stained cells per mm^2. CC, corticosteroid; CGNP, cerebellar granule neuron progenitor.

neurodevelopmental and/or neurodegenerative disorders (Bose et al., 2010; Austdal et al., 2016).

The effect of prenatal GCs on the reduction of Purkinje cell dendritic outgrowth in P22, P52 and P82 rats has been shown (Pascual et al., 2017). Our analysis of Purkinje cells by AgNOR impregnation showed disordered cells, with an irregular arrangement, and an increase in the number of nucleoli and NORs in P12 CC rats; that is, the transcriptional level was enhanced. This increase can be interpreted as damage generated by CC, with the consequent effort of the Purkinje cells to counteract it because nucleolar disturbances are frequently associated with cellular stress (Mayer and Grummt, 2005).

To evaluate the effect of a decrease in CGNP on migration and differentiation, we used the Golgi-Kopsch method. Since the cerebellums of P12 rats from both the CC group and control group showed no differences in cell migration and differentiation of the lineage coming from CGNP, we included P0 and P6 rats, which includes ages when most granule cells have not colonised the IGL. Usually, proliferating CGNP cells leave the cell cycle and subsequently start differentiation, axonal growth, and migration (Wechsler-Reya and Scott, 1999). Nevertheless, our results suggest a premature abandonment of the cell cycle, with consequent disturbances in cell migration and differentiation of granule neurons. Because only P6 rats of the CC group showed an increase in CGNP morphology in the EGL, this seems to be a rebound in the proliferation of CGNP caused by CC. However, P6 rats also showed a decrease in migratory stages across the ML and the PCL and an increase in young granule cell stages in the IGL. However, despite the rebound, the total impregnated cell number of lineages derived from CGNP was not compensated. In the same way, Scheepens et al. (2003) found that prenatal single doses of betamethasone result in decreases in brain size, which is not compensated by a rebound in neural proliferation of both the brain and cerebellum.

Morphometric analysis of Bergmann glia through the Golgi-Kopsch method revealed a decrease in soma growth and reduction in the number of processes in the CC group. This suggests a global detriment in glia branching by prenatal CC administration. A reduction in the processes density of the Bergmann glia was shown in infants of preterm birth (Baud and Gressens, 2011).

In this work, we observed a decrease in cerebellar CGNP accompanied by advancement in cell migration and differentiation of granule microneurons in the experimental group. We also observed negative effects on Bergmann glia and Purkinje cell development during the early postnatal stage. These observations agree with studies of preterm delivery infants. For this reason, we suggest that prenatal CC leads to cellular stress which can cause premature development or signals of maturation and aging of different cerebellar neuron cell types. In conclusion, silver staining allowed us to study the effects of prenatal CC administration on nervous system development, a process with a strict temporal pattern. Moreover, our experimental model in rats supports the risk of prenatal CC administration for offspring.

Acknowledgements. We thank Dafne E. Bonilla Cerquedo for performed imagings and Emilio Espinoza Simón for critical reading of the manuscript and suggestions.

Conflicts of interest. The authors declare that they have no conflict of interest.

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Accepted December 17, 2020