ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Research paper Acta Neurobiol Exp 2014, 74: 415–423

Single course of antenatal betamethasone produces delayed changes in morphology and calbindin-D28k expression in a rat's cerebellar Purkinje cells

Rodrigo Pascual*, Martina Valencia, Sebastián Larrea, and Carlos Bustamante

Laboratorio de Neurociencias, Escuela de Kinesiología, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile, *Email: rodrigo.pascual@ucv.cl

In the current study, we analyzed the impact of antenatal betamethasone on macroscopic cerebellar development, Purkinje cell morphology and the expression of the neuroprotective protein calbindin-D28k. Pregnant rats (Sprague-Dawley) were randomly divided into two experimental groups: control (CONT) and betamethasone-treated (BET). At gestational day 20 (G20), BET dams were subcutaneously injected with a solution of 0.17 mg kg^{-1} of betamethasone, while CONT animals received a similar volume of saline. At postnatal days 22 (P22) and P52, BET and CONT offspring were behaviorally evaluated, and the cerebella were histologically and immunohistochemically processed. Animals that were prenatally treated with a single course of betamethasone exhibited long-lasting behavioral changes consistent with anxiety-like behavior in the open-field test, together with (1) reduced cerebellar weight and volume, (2) Purkinje cell dendritic atrophy, and (3) an overexpression of calbindin-D28k. The current results indicate that an experimental single course of betamethasone in pregnant rats produces long-lasting anxiety-like behaviors, together with macroscopic and microscopic cerebellar alterations.

Key words: antenatal betamethasone, Purkinje cell development, fetal programming, calbindin-D28k

INTRODUCTION

It has been shown that premature infants have a greater risk for suffering alterations in cerebellar maturation, in close association with mild motor and cognitive abnormalities (Allin et al. 2001, Shah et al. 2006, Spittle et al. 2010). Furthermore, it has recently been shown that the postnatal administration of synthetic glucocorticoids (GCs) in preterm babies is associated with a reduction in cerebellar, but not cerebral, growth (Tam et al. 2011). Similar results were observed in fetal sheep, where the cerebellar size was significantly reduced by single and repeated courses of antenatal betamethasone (0.5 mg kg^{-1}), especially in the vermal surface (Huang et al. 1999). This increased cerebellar vulnerability may be explained, at least in part, by two facts. First, during the last trimester of gestation and the early postnatal months, human cerebellar tissue exhibits an accelerated volumetric expansion and foliation (Lavezzi et al. 2006, Bolduc et al. 2011, Ginath et al. 2013). Second, during perinatal ages, cerebellar tissue expresses a higher GC receptor density (Pavlik and Buresová 1984).

At the histological level, only one experimental study has showed that rats exposed to a single course of antenatal betamethasone in a clinically equivalent dose (0.17 mg.kg^{-1}) exhibited significant cerebellar cell proliferation reductions compared to age-matched control animals (Scheepens et al. 2003). Consistent with these data, in our laboratory, we demonstrated that animals subjected to chronic prenatal stress exhibit a significant long-term dendritic impairment in vermal Purkinje cells, which is evident only when animals reach adulthood (Pascual et al. 2010). Although these data suggest that stress-induced increases in endogenous GCs could exert a long-term deleterious impact on cerebellar tissue, it is unknown whether GCs are directly responsible for this protracted Purkinje cell

Correspondence should be addressed to R. Pascual
Email: rodrigo.pascual@ucv.cl

Received 14 April 2014, accepted 08 October 2014

impairment. Thus, in the present study, we assessed whether synthetic GCs (betamethasone), administered in a clinically equivalent dose and at an age equivalent to a premature infant (gestational day 20) (Scheepens et al. 2003, Clancy et al. 2007), produce long-term alterations in (1) cerebellar weight and volume, (2) Purkinje cell dendritic morphology using Golgi-Cox stained tissue, and (3) the immunohistochemical expression of the neuroprotective protein calbindin-D28K (CAD; Wójcik et al. 2013). We selected this protein for three main reasons: first, it is the major calcium sequestering protein in Purkinje cells (Bastianelli 2003); second, its expression is associated with changes in the levels of GCs (Iacopino and Christakos 1990, Krugers et al. 1995); and third, it is related to cerebellar function because changes in its expression may affect motor behavior (Airaksinen et al. 1997, Barski et al. 2003). In addition, because vermal Purkinje cells are involved in motor and emotional regulation (Sacchetti et al. 2009), we quantified the antenatal betamethasone-induced anxiety-like behaviors evaluated in an open-field test (Rebolledo-Solleiro et al 2013).

METHODS

Laboratory Animals

Animals were treated and housed in accordance with the "Principles of Laboratory Animal Care"(NIH publication N° 86-23, revised 1985), and the experimental protocols received approval from the local animal ethics committee.

Thirty Sprague-Dawley multiparous female rats, purchased from the animal vivarium of Faculty of Chemistry and Pharmacy at the University of Chile, were used. All animals were housed under controlled environmental conditions: temperature (20±1°C) and day-night cycle (12:12 light-dark), with food and water ad libitum. Once they mated (1 female with 1 male per cage), females (n=30) were placed in individual cages (45×25×20 cm), and the gestational day 0 (G0) was determined by the presence of sperm detected by vaginal smears. Pregnant rats were randomly classified into 2 study groups: control-saline and betamethasone-treated. Then, offspring were divided into 4 experimental groups: (1) control-saline, evaluated at 22 postnatal days (CONT-P22, n=13), (2) betamethasone, evaluated at 22 postnatal days (BET-P22, n=9), (3) control-saline, evaluated at 52 postnatal days (CONT-P52, n=12), and (4) betamethasone, evaluated at 52 postnatal days (BET-P52, n=13). To avoid gender-related influences, the behavioral and neuronal assessments were conducted only in male animals (60–70% of male rats were randomly selected from each litter).

Drug treatment

Mothers of BET-P22 and BET-P52 groups were given a single curse of betamethasone (0.17 mg kg^{-1}, subcutaneously, dorsal neck region) (Cidoten®, Schering-Plough, Inc., Santiago, Chile) on gestational day 20 (G20), separated by an 8-hour interval. We used this procedure because it is equivalent to that employed in the obstetric clinic (2 doses every 24 hours, 12 mg c/u) (Scheepens et al. 2003). G20 was chosen because it corresponds to an approximate ontogenetic stage of a pre-term human (24–32 weeks gestation) (Lavezzi et al. 2006). Mothers of CONT-P22 and CONT-P52 received an equal volume (1 ml) of normal saline (see timeline in Fig. 1).

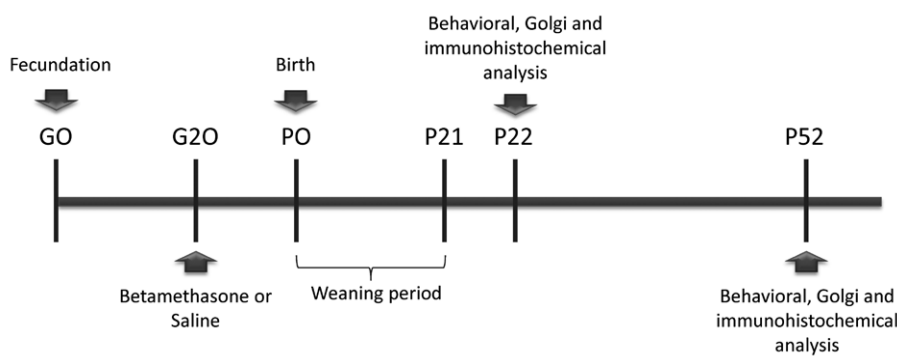

Fig. 1. Time line showing the experimental design. (G0), (G20) gestational age (days); (P0), (P21), (P22), (P52) postnatal age (days).

Behavioral testing

On postnatal day 22 (P22) or P52, each animal in the control (saline) and betamethasone groups was placed in the central quadrant of the device (black wooden surface, 60×60 cm bounded by a wall 32 cm height for P22 animals and 90x90x32 cm for P52 animals). Motor activity was quantified using two parameters: the average time spent and the number of entries in the center of the arena over 5 minutes. These variables constitute good indicators of behaviors consistent with anxiety-like disorders (Rebolledo-Solleiro et al. 2013). These behaviors were recorded by videotape and analyzed using the Any-maze 4.60 (Stoelting Co.,Wood Dale, USA).

All behavioral analyses were conducted between 09:00 AM and 03:00 PM, and the apparatus was carefully cleaned with 5% ethanol between each analysis. The animals were taken to the behavioral assessment room 10 minutes prior to behavioral testing to allow habituation to the new environment. In all behavioral tests described, white noise was used (background; 60 decibels), thereby minimizing any sudden noise that may have interfered with the behavior of the animal. All behaviors were analyzed by the same investigator, who was blinded to the experimental condition.

Macroscopic, Golgi-Cox histology and immunohistochemical analysis

Following behavioral assessment, all male animals (P22–P52) were anesthetized with isoflurane or sodium pentobarbital (Merck, Darmstadt, Germany) lethal dose. Once a deep anesthetic state was confirmed, intracardiac perfusion was performed (0.9% NaCl and then paraformaldehyde 4%). Cerebella were removed, macroscopically assessed in the fresh state (weight and volume using a digital balance and Golgi-Cox solution displacement in a graduated glass cylinder, respec-

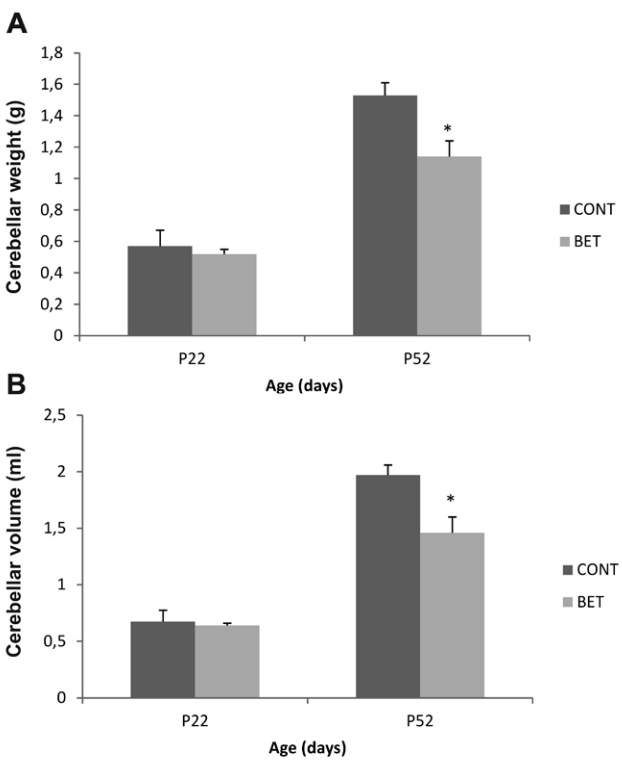

Fig. 2. (A) Fresh cerebellar weight at postnatal day 22 (P22) in CONT and BET groups (t_9=0.58, ns) and at P52 in CONT and BET groups (t_{10}=2.95, **P*<0.05). (B) Fresh cerebellar volume at P22 in CONT and BET groups (t_9=0.35, ns) and at P52 in CONT and BET groups (t_{10}=3.04, **P*<0.05). Data are the means ± SEM.

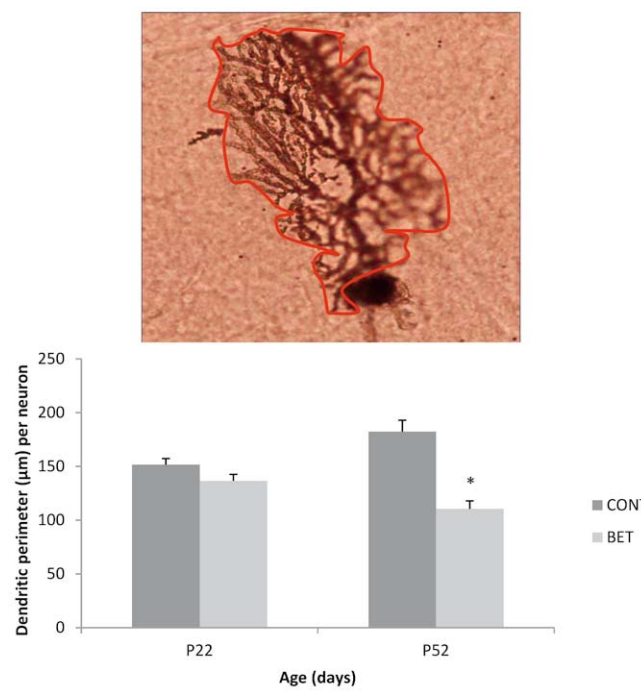

Fig. 3. (Top) example of dendritic tree perimeter quantified using the Micrometrics SE Premium V-2.8 software (red line). (Bottom) dendritic perimeter quantified in Golgi-Cox-stained cerebellar Purkinje cells (lobule IX). (CONT) control group; (BET) betamethasone group; (P22)–(P52) postnatal days 22 (t_{99}=1.03, ns) and 52 (t_{51}=3.701, **P*<0.05), respectively. Data are the means ± SEM.

tively) and post-fixed for one hour and stored in 30% sucrose at 4°C for 7 days for cryoprotection. Finally, the cerebellar vermis was sectioned (20 and 80 µm for immunohistochemical and Golgi-Cox procedures, respectively) using a Microm HM525 Thermo-Scientific cryostat. In all cases, the cerebellar lobule IX of each section was digitalized to measure neuronal variables. We selected this region because, according to the anatomical and functional connections, it is associated with motor and emotional behaviors (Strata et al. 2011).

To determine the effect of drug administration on dendritic expansion of vermal Purkinje cells, cerebellar vermal sections were processed with Golgi-Cox staining. Briefly, the cerebella were removed and immediately immersed in 50 ml of Golgi ($K_2Cr_2O_7$, $HgCl_2$, and K_2CrO_4, all at 5%; Merck) for 45 days at room temperature and in complete darkness. Subsequent, brain slices were placed in a sucrose solution (30%) for at least one week and were sectioned in the cryostat (Microm HM525, Thermo Scientific, Microm GMBH, Walldorf, Germany). Subsequently, Golgi-Cox sections were washed in distilled water, immersed in a solution of oxalic acid and potassium metabisulphite (5%, Merck, Darmstadt, Germany), dehydrated in alcohol-acetone and alcohol-ether (50–50%, PA, Merck, Darmstadt, Germany), embedded in α-terpineol, mounted in code slides with Canadian balsam (Fluka, Sigma-Aldrich, Steinheim, Germany) and coverslipped. Sections were observed under a light microscope (Olympus CCD 5.0) at 400×. Purkinje cells that fulfilled the criteria used in Pascual and coworkers (2010) and Pascual and Bustamante (2013) were selected and captured using a camera attached to the microscope. A total of 154 Purkinje cells were analyzed from the following time points: P22 (CONT-P22, n=49; BET-P22, n=52); P52 (CONT-P52, n=33; BET-P52, n=20). The dendritic tree perimeter of each selected Purkinje cell was quantified by drawing a polygon joining the distal-most tips of dendrites using the Micrometrics SE Premium V-2.8 software.

The expression of the histological localization of CAD was studied in the cerebellar vermal Purkinje cells by immunohistochemistry. Sections that were previously attached to the slide were washed in PBS twice for 10 minutes each at 90 rpm and then incubated with 0.5% H_2O_2 (Merck , Darmstadt, Germany) for 30 minutes at room temperature. After two additional washes with PBS (1X), we proceeded to block with BSA (Sigma) 3% Triton X-100 (Sigma) 0.4% for one hour. The primary antibody used was anti-calbindin-D28k (1/1000, ab1778, Millipore, Sigma, Temecula, USA), which was incubated in blocking solution overnight at room temperature under agita-

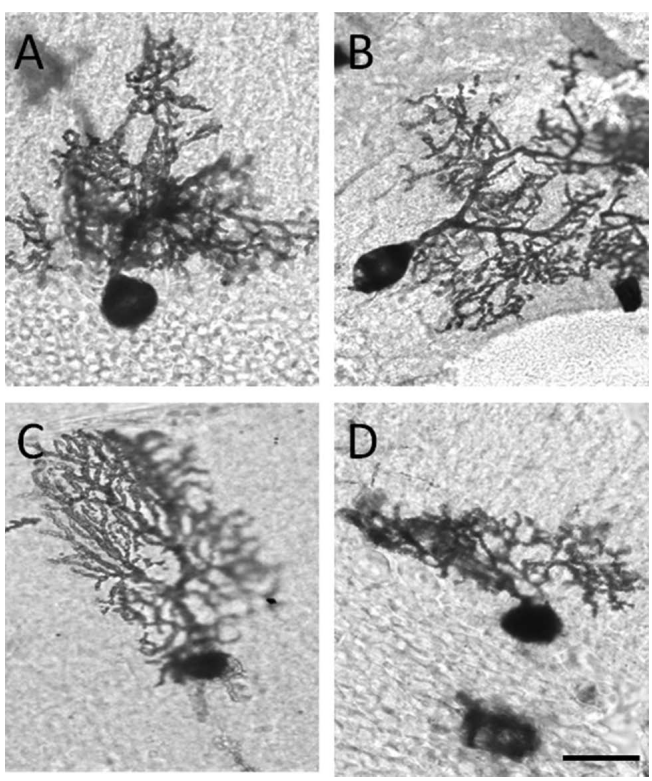

Fig. 4. Representative cerebellar Purkinje cells stained with the Golgi-Cox procedure. (A), (C) control animals at 22 and 52 postnatal days, respectively; (B), (D) betamethasone-treated animals at 22 and 52 postnatal days, respectively. Sale bar is 20 µm.

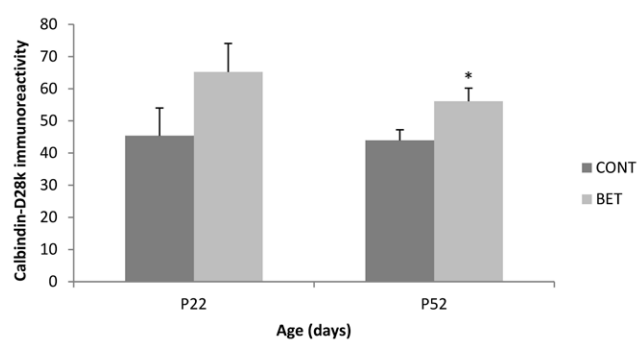

Fig. 5. Calbindin-D28k immunoreactivity. CONT and BET groups at P22: (t_8=1.79, ns) and at P52 (t_{13}=2.74, *P<0.05). (CONT) control group; (BET) betamethasone group. Data are the means ± SEM.

tion (40 rpm). The tissue was then washed three times with PBS (1×) and incubated in the medium 1.5% BSA and Triton X-100 0.2% for two hours at room temperature and 40 rpm agitation. Then, the tissue was washed again (three times) with PBS (1X). The secondary antibody, an Avidin-Biotin peroxidase complex associated (Vectastain® Elite ABC Kit, Vector Laboratories Inc., Brulingame, USA), was prepared in 1.5% BSA and Triton X-100 for one hour and added to the substrate coupled with diaminobenzidine for 20 minutes without stirring to visualize the labeled protein (ImmPACT TM DAB Peroxidase Substrate, Vector Laboratories Inc., Brulingame, USA). Finally, the sections were washed with distilled water for 10 seconds. The sections were immediately mounted on slides, dried in the air, and then covered with Entellan (Merck) and coverslipped. The cerebellar vermal sections (P22, n=10; P52, n=15) were coded and observed using a Motic BA210-Tech Lab microscope. We evaluated CAD-immunoreactivity (arbitrary absolute values) using the grey scale image by the Image-J software.

Statistical analysis

For the analysis of behavioral, morphological and immunohistochemical data (saline P22 *versus* betamethasone P22; and saline P52 *versus* betamethasone P52) *t*-test for independent groups were used (STATA 9.1 software). The results are presented as the mean ± SEM. Differences at $P<0.05$ were considered significant.

RESULTS

Cerebellar weight and volume

As shown in Figure 2, cerebellar weight (g) and volume (ml) of infant animals (postnatal day 22, P22) that were exposed prenatally to betamethasone in doses equivalent to those used in perinatal medicine showed no macroscopic changes compared with the saline controls. However, when they reached adolescence (P52), cerebellar weight and volume of betamethasone-treated animals showed significant reductions (−19.8%, t_{10}=2.95, $P<0.05$ and −10.3%, t_{10}=3.04, $P<0.05$, respectively) compared to the age-matched control animals.

Purkinje cell dendritic perimeter

Consistent with macroscopic data, Purkinje cells of infant animals (postnatal day 22, P22) that were exposed prenatally to betamethasone showed no morphological changes compared with the saline controls (Fig. 3). However, when they reached adolescence (P52), Purkinje cell dendritic perimeter showed a significant reduction (−39.4%; t_{51}=3.701, $P<0.05$) compared to the age-matched control animals, indicating that the use of synthetic GCs has a protracted deleterious impact during later developmental stages. Representative Golgi-Cox stained Purkinje cells are shown in Figure 4.

Calbindin-D28k (CAD) expression

As shown in Figure 5, although P22 betamethasone-treated animals showed an increase in the CAD immunoreactivity per cerebellar vermal lobule IX this effect did not reach significance. However, this trend was significant at P52 compared to the age-matched saline animals (−28.1%, t_{13}=2.74, $P<0.05$). Figure 6 shows representative regions of cerebellar lobule IX stained by CAD.

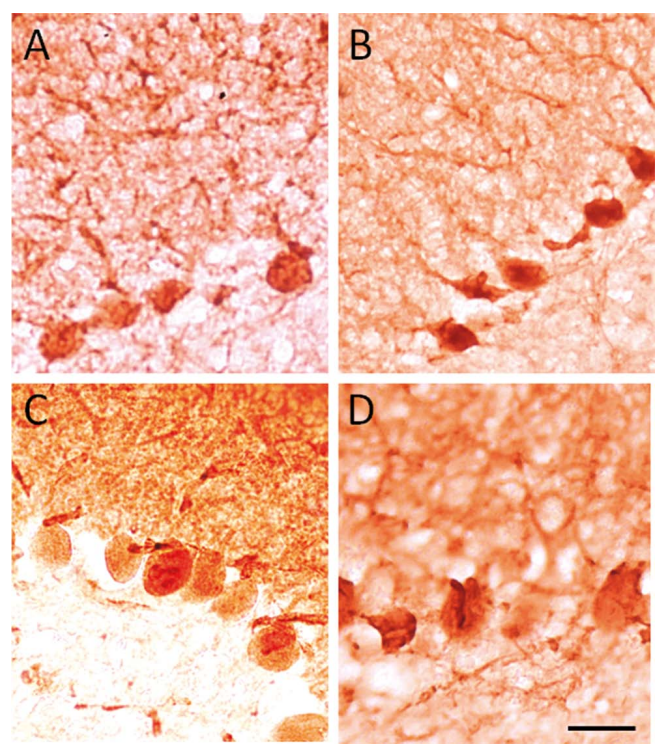

Fig. 6. Representative photomicrographs of lobule IX stained with anti-calbindin-D28k. (A), (C) control group at 22 and 52 postnatal days; (B), (D) betamethasone group at 22 and 52 postnatal days, respectively. Scale bar is 20 µm.

Behavioral testing

Consistent with the morphological and immunohistochemical data, betamethasone-treated animals showed behavioral changes only during adolescence (P52). As shown in Figure 7A, P52 animals showed a significant reduction in locomotor activity (number of entries) in the central zone of the open field test compared to the age-matched control animals (−71.2%, t_{26}=4.63, P<0.001). Similarly, the time spent in the central zone of the arena showed a significant reduction in exploratory behavior at P52 (−57.6%, t_{26}=2.43, P<0.05; Fig. 7B).

Body weight

Consistent with previous clinical and preclinical studies, body weight gain was significantly lower in the betamethasone-treated animals at both P22 (−10.5%, t_{20}=5.41, P<0.05) and P52 (−7.1% t_{23}=4.69, P<0.05; Fig. 8).

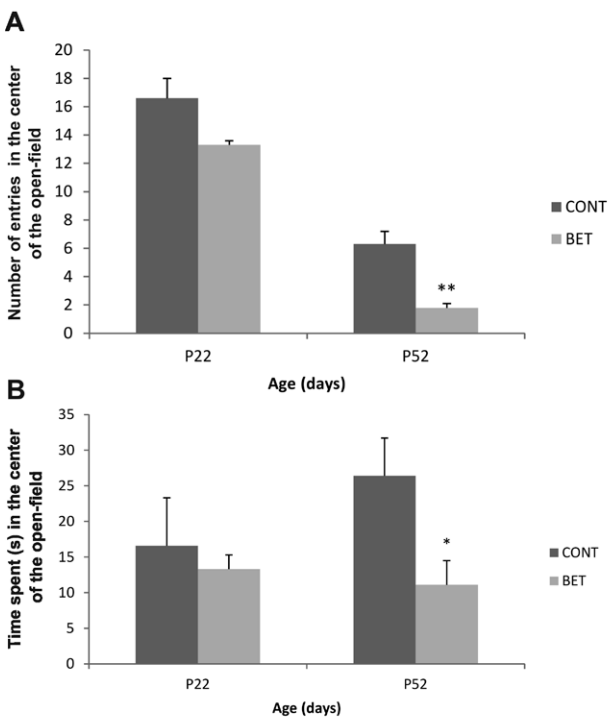

Fig. 7. Behavioral evaluations in the open-field test. (A) Number of entries in the central zone at postnatal day 22 (P22) in CONT and BET groups (t_{20}=1.86, ns) and at P52 in CONT and BET groups (t_{26}=4.63, **P<0.001). (B) Time spent in the central zone at P22 in CONT and BET groups (t_{20}=0.55, ns) and at P52 in CONT and BET groups (t_{26}=2.43, *P<0.05). Data are the means ± SEM.

DISCUSSION

This study found that antenatal betamethasone administration of an equivalent clinical dose produced significant changes in cerebellar growth, dendritic perimeter and CAD expression in vermal Purkinje cells, in close association with changes in locomotor behavior in the open-field test. Of note, the deleterious impact of betamethasone on neurobehavioral variables was significant when animals reached adolescent ages.

This is the first study to report that a clinically equivalent single course of antenatal betamethasone (0.17 mg kg^{-1}) impaired neuronal morphology in the cerebellar tissue. Importantly, this deleterious impact was evident when animals reached sexual maturity, but not during the early post weaning period, suggesting that betamethasone exerts a protracted effect on Purkinje cell morphology. This effect is entirely consistent with our previous studies, in which we showed that prenatal stress produced a similar reduction in the dendritic arborization of Purkinje cells that became apparent only when the animals reached adulthood (Pascual et al. 2010). Although that study did not assess the maternal corticosterone levels, according to the data obtained in the current work, it seems likely that the mechanism involved in the dendritic deterioration may be the action of GCs on the intracellular receptors that act genomically and non-genomically on immature Purkinje cells. This increased vulnerability may be explained by the fact that cerebellar tissue expresses higher GC receptors, especially during pre- and postnatal development (Pavlik and Burešová 1984). In addition, it has been suggested that the deleterious effects of synthetic glucocorticoids, including betamethasone, may be induced indirectly by fetal brain hemodynamic changes. In fact, animals (ewes) prenatally exposed to betamethasone showed a significant reduction in the carotid blood flow associated with an increased oxidative damage in the fetal hippocampus compared to the age-matched control animals (Miller et al. 2007).

It then seems likely that treatment with GCs, despite the respiratory status benefits observed in preterm infants, could alter cerebellar development at the microscopical level. Indeed, in a recent study Tam and colleagues (2011) using neuroimaging analysis by nuclear magnetic resonance reported that the use of synthetic GCs (mainly dexamethasone) in preterm

infants was associated with a significant lower gross cerebellar, but not cerebral, development. Although the antenatal administration of betamethasone was not associated with significant cerebellar alterations, Tam's group (2011) observed a strong relationship between perinatal administration of hydrocortisone or dexamethasone and lower volumetric cerebellar outgrowth. While the study of Tam and others (2011) did not observe an association between prenatal administration of betamethasone and the macroscopic development of the cerebellum, microscopic alterations cannot be excluded in betamethasone-treated preterm babies, as experimentally demonstrated in the current study. Furthermore, our current cerebellar weight and volume reductions detected in BET groups are consistent with results showing that three courses of corticosterone administration on pregnant rats (17, 18, and 19 days of gestation) significantly reduced the cerebellar weight and cortical cerebellar lamination (Velazquez and Romano 1987). In addition, Arahuetes and coauthors (1991) showed that maternal BET treatment increased brain lipid concentration associated with enhanced brain myelination, suggesting that glucocorticoids could alter the normal fetal brain development.

Moreover, the Purkinje cells of animals exposed to antenatal betamethasone showed a significant increase in the expression of CAD with respect to their age-matched controls. Similar to the morphological data, the overexpression of CAD reaches significance in the late stages of development. Because Purkinje cells usually increase CAD expression to minimize intracellular calcium transients, the overexpression detected only in those animals treated with betamethasone can be interpreted as a neuroprotective mechanism triggered by the Purkinje cells in an attempt to reduce cell toxicity secondary to calcium overload. This interpretation is based on previous studies conducted in our laboratory, in which the post-weaning stress induced by social isolation was related to a significantly increased level of CAD cell expression in medial prefrontal neurons (Pascual et al. 2013). Furthermore, CAD increases in betamethasone-treated animals may be related to the fact that prenatal synthetic GCs can increase calcium permeability in the mitochondrial membranes of cerebellar neurons, rendering them more vulnerable to oxidative stress (Ahlbom et al. 2000). Because Golgi-Cox stained Purkinje cells showed a significant dendritic atrophy despite higher levels of CAD expression in the present study, it is possible that this protracted neuroprotective mechanism may not have been sufficient to offset the deleterious impact of betamethasone. In addition, since the deleterious effect of BET on Purkinje cell dendritic phenotype was significant only at P52, it is possible to speculate that the late calbindin-D28k overexpression is triggered by BET-induced changes in Purkinje cell dendritic morphology (outgrowth stunted and/or retraction); hence, the compensatory Purkinje cell-CAD expression at P22 was probably not necessary.

Furthermore, betamethasone-exposed animals showed significant impairments in behavioral motor activity evaluated in the open-field test that, consistent with histological variables, were significant only in the late ontogenetic stages. The diminished motor exploratory behavior in the central perimeter of the arena observed in animals treated with betamethasone could be interpreted as anxiety-like behaviors because the exploration in the open central zone indicates that animals are exposed to a ethologically risky open space (without side walls). Although we did not discard potential motor disturbances, the described anxiety-like behavior may be related to Purkinje cell morphological alterations showed by animals that were antenatally treated with betamethasone. This interpretation is based on the fact that Purkinje cells located in the posterior cerebellar medial lobule IX establish numerous connections with the limbic system, including the amygdala, the hypothalamus, the hippocampus, and the cingulate and orbitofrontal cortices (Sacchetti et al. 2009, Strata et al. 2011). Therefore, inadequate function of these connections may alter the function of one

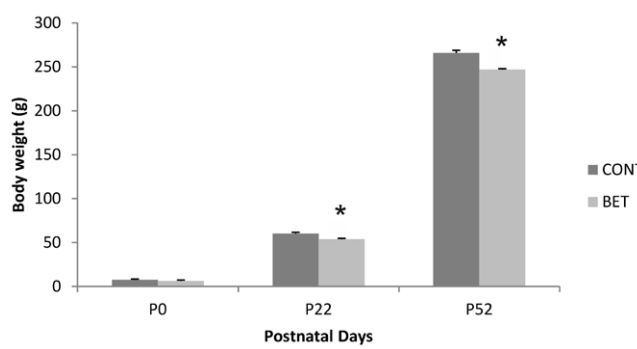

Fig. 8. Body weight (g) of the offspring animals. (CON) control group; (BET) betamethasone group; (P0), (P22), (P52) postnatal days 0, 22 and 52. Data are the means ± SEM *P<0.05 (t_{20}=5.41) and *P<0.05 (t_{23}=4.69) at 22 and 52 postnatal days, respectively.

or more limbic structures, producing behaviors that are consistent with anxiety-like disorders.

Although we do not know why cerebellar impairment occurs only at late stages of postnatal life, it is possible to speculate that endogenous glucocorticoids act as nuclear transcription factors may alter the genetic expression later in life. This conjecture is consistent with the so-called fetal programming hypothesis of Barker, where he argues that hormonal and other undetermined systemic changes during prenatal life constitute a risk factor in the genesis of certain diseases during adulthood (e.g., type II diabetes, dyslipidemias, obesity and heart disease, reviewed in Rinaudo and Wang 2012).

Despite the consistency of our data with other studies (Velazquez and Romano 1987, Pascual et al. 2010, Tam et al. 2011) data obtained in the present study should be interpreted with caution. First, there may be differences in the degree of vulnerability of the cerebellum of the rat versus other more evolved animals, i.e. monkeys and humans; second, the brain of newborn rats is slightly more immature than that of primates, including humans (Clancy et al. 2007). Finally, although the dose of betamethasone prenatally administered is equivalent to that used in the human clinical practice (Scheepens et al. 2003), there may be unknown pharmacokinetics and/or pharmacodynamics variables that could influence the neuronal and behavioral data obtained in our current and other studies.

CONCLUSIONS

According to our morphological, immunohistochemical and behavioral data, antenatal betamethasone administered in clinical doses (0.17 mg kg^{-1}) and at equivalent ontogenetically stages (gestational day 20), produced lasting changes in Purkinje cell dendritic morphology, CAD expression, and motor behavior consistent with anxiety-like disorders. Finally, it is possible that the neurobehavioral sequel described in preterm children prenatally treated with synthetic GCs (Spinillo et al. 2004) may be related to undetected microscopic cerebellar impairments, similar to the findings described in the present study.

Furthermore, although in the current study we administered a similar dose of betamethasone used in perinatal medicine (Scheepens et al. 2003, Bruschettini et al. 2006) and that the rat is currently a good mammalian animal model, it is necessary to keep in mind the potential species-specific differences when extrapolates animal data to the human condition.

ACKNOWLEDGMENT

The authors thank the DII PUCV-department facilities for English style revision and financial support by grant PUCV-VRIEA 037.482-2013.

REFERENCES

Ahlbom E, Gogvadze V, Chen M, Celsi G, Ceccatelli S (2000) Prenatal exposure to high levels of glucocorticoids increases the susceptibility of cerebellar granule cells to oxidative stress-induced cell death. Proc Natl Acad Sci U S A 97: 14726–14730.

Airaksinen MS, Eilers J, Garaschuk O, Thoenen H, Konnerth A, Meyer M (1997) Ataxia and altered dendritic calcium signaling in mice carrying a targeted null mutation of the calbindin-D28k gene. Proc Natl Acad Sci U S A 94: 1488–1493.

Allin M, Matsumoto H, Santhouse AM, Nosarti C, Al Asady MH, Stewart AL, Rifkin L, Murray RM (2001) Cognitive and motor function and the size of the cerebellum in adolescents born very pre-term. Brain 124: 60–66.

Arahuetes RM, Carretero V, Diebold Y, Rua C (1991) Effects of maternal bilateral adrenalectomy and betamethasone administration on fetal rat encephalic development. Biol Neonate 59: 303–313.

Barski JJ, Hartmann J, Rose CR, Hoebeek F, Mörl K, Noll-Hussong M, De Zeeuw CI, Konnerth A, Meyer M (2003) Calbindin in cerebellar Purkinje cells is a critical determinant of the precision of motor coordination. J Neurosci 23: 3469–3477.

Bastianelli E (2003) Distribution of calcium-binding proteins in the cerebellum. Cerebellum 2: 242–262.

Bolduc ME, Du Plessis AJ, Evans A, Guizard N, Zhang X, Robertson RL, Limperopoulos C (2011) Cerebellar malformations alter regional cerebral development. Dev Med Child Neurol 53: 1128–1134.

Bruschettini M, van den Hove DL, Timmers S, Welling M, Steinbusch HP, Prickaerts J, Gazzolo D, Blanco CE, Steinbusch HW (2006) Cognition- and anxiety-related behavior, synaptophysin and MAP2 immunoreactivity in the adult rat treated with a single course of antenatal betamethasone. Pediatr Res 60: 50–54.

Clancy B, Finlay BL, Darlington RB, Anand KJ (2007) Extrapolating brain development from experimental species to humans. Neurotoxicology 28: 931–937.

Ginath S, Lerman-Sagie T, Haratz Krajden K, Lev D, Cohen-Sacher B, Bar J, Malinger G (2013) The fetal vermis, pons and brainstem: normal longitudinal development as shown by dedicated neurosonography. J Matern Fetal Neonatal Med 26: 757–762.

Huang WL, Beazley LD, Quinlivan JA, Evans SF, Newnham JP, Dunlop SA (1999) Effect of corticosteroids on brain growth in fetal sheep. Obstet Gynecol 94: 213–218.

Iacopino AM, Christakos S (1990) Corticosterone regulates calbindin-D28k mRNA and protein levels in rat hippocampus. J Biol Chem 265: 10177–10180.

Krugers HJ, Medema RM, Postema F, Korf J (1995) Region-specific alterations of calbindin-D28k immunoreactivity in the rat hippocampus following adrenalectomy and corticosterone treatment. Brain Res 696: 89–96.

Lavezzi AM, Ottaviani G, Terni L, Matturri L (2006) Histological and biological developmental characterization of the human cerebellar cortex. Int J Dev Neurosci 24: 365–371.

Miller SL, Chai M, Loose J, Castillo-Meléndez M, Walker DW, Jenkin G, Wallace EM (2007) The effects of maternal betamethasone administration on the intrauterine growth-restricted fetus. Endocrinology 148: 1288–1295.

Pascual R, Ebner D, Araneda R, Urqueta MJ, Bustamante C (2010) Maternal stress induces long-lasting Purkinje cell developmental impairments in mouse offspring. Eur J Pediatr 169: 1517–1522.

Pascual R, Bustamante C (2013). Early postweaning social isolation but not environmental enrichment modifies vermal Purkinje cell dendritic outgrowth in rats. Acta Neurobiol Exp (Wars) 73: 387–393.

Pascual R, Zamora-León P, Bustamante C (2013) Selegiline (deprenyl) decreases calbindin-D28k expression in cortical neurons of rats socially deprived during the postweaning period. Int J Dev Neurosci 31: 145–149.

Pavlík A, Buresová M (1984) The neonatal cerebellum: the highest level of glucocorticoid receptors in the brain. Brain Res 314: 13–20.

Rebolledo-Solleiro D, Crespo-Ramírez M, Roldán-Roldán G, Hiriart M, Pérez de la Mora M (2013) Role of thirst and visual barriers in the differential behavior displayed by streptozotocin-treated rats in the elevated plus-maze and the open field test. Physiol Behav 120: 130–135.

Rinaudo P, Wang E (2012) Fetal programming and metabolic syndrome. Annu Rev Physiol 74: 107–130.

Sacchetti B, Scelfo B, Strata P (2009) Cerebellum and emotional behavior. Neuroscience 162: 756–762.

Shah DK, Anderson PJ, Carlin JB, Pavlovic M, Howard K, Thompson DK, Warfield SK, Inder TE (2006) Reduction in cerebellar volumes in preterm infants: relationship to white matter injury and neurodevelopment at two years of age. Pediatr Res 60: 97–102.

Scheepens A, van de Waarenburg M, van den Hove D, Blanco CE (2003) A single course of prenatal betamethasone in the rat alters postnatal brain cell proliferation but not apoptosis. J Physiol 552: 163–175.

Spinillo A, Viazzo F, Colleoni R, Chiara A, Maria Cerbo R, Fazzi E (2004) Two-year infant neurodevelopmental outcome after single or multiple antenatal courses of corticosteroids to prevent complications of prematurity. Am J Obstet Gynecol 191: 217–224.

Spittle AJ, Doyle LW, Anderson PJ, Inder TE, Lee KJ, Boyd RN, Cheong JL. (2010) Reduced cerebellar diameter in very preterm infants with abnormal general movements. Early Hum Dev 86: 1–5.

Strata P, Scelfo B, Sacchetti B (2011) Involvement of cerebellum in emotional behavior. Physiol Res 60 Suppl 1: S39–48.

Tam EW, Chau V, Ferriero DM, Barkovich AJ, Poskitt KJ, Studholme C, Fok ED, Grunau RE, Glidden DV, Miller SP (2011) Preterm cerebellar growth impairment after postnatal exposure to glucocorticoids. Sci Transl Med 3: 105ra–105. [doi: 10.1126/scitranslmed.3002884]

Velazquez PN, Romano MC (1987) Corticosterone therapy during gestation: effects on the development of rat cerebellum. Int J Dev Neurosci 5: 189–194.

Wójcik S, Łuczyńska A, Dziewiatkowski J, Spodnik E, Ludkiewicz B, Moryś J (2013) Expression of the calcium-binding proteins in the central, medial and cortical nuclei of the rabbit amygdaloid complex during postnatal development. Acta Neurobiol Exp (Wars) 73: 260–279.