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Prenatal glucocorticoid administration persistently increased the immunohistochemical expression of type-1 metabotropic glutamate receptor and Purkinje cell dendritic growth in the cerebellar cortex of the rat

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Abstract

Several studies have indicated that abnormal prenatal changes in the circulating glucocorticoids (GCs), induced by either maternal stress or exogenous GC administration, significantly alter the development of Purkinje cells (PCs). Among the suggested mechanisms that could mediate this GC-dependent PC susceptibility are changes in the expression of type-1 metabotropic glutamate receptors (mGluR1). In the current study, we analyzed whether a single course of prenatally administered betamethasone phosphate (BET) in pregnant rats increased the immunohistochemical expression of mGluR1 in PCs and decreased PC dendritic growth. The data obtained showed that *in utero* BET exposure resulted in a significant immunohistochemical overexpression of mGluR1 and a significant reduction in Purkinje cell dendritic outgrowth during postnatal life.

Keywords: prenatal stress, Purkinje cell, mGluR1, synthetic glucocorticoids.

Introduction

Preclinical studies have shown that prenatal stress produces long-term postnatal changes in cerebellar Purkinje cell (PC) dendritic growth [1], which can be mimicked by a single course of prenatal synthetic glucocorticoid (GC) administration [2]. Although the pathophysiological mechanisms by which the GCs levels alter PC development are unknown, at least one potential cause may be the abnormal interaction among circulating GCs, type-1 metabotropic glutamate receptors (mGluR1) expression and intracellular calcium changes. For example, it has been observed that the exogenous application of synthetic GCs (sGCs) induced significant changes in the free calcium levels of hypothalamic, hippocampal and HT4 neuroblastoma cells [3–5]. Similarly, in a previous study, we showed that the antenatal administration of sGCs significantly increased the immunohistochemical expression of the PC calcium-sequestering protein calbindin-D28k, in close association with dendritic branching impairments [2]. This increase in calbindin-D28k expression was interpreted as a compensatory mechanism to avoid the toxic increases in the cytosolic free calcium levels.

Purkinje cell dendrites express a high density of the subtype GluR1 coupled with inositol triphosphate (IP_3)/Ca^{2+} signal transduction cascades [6, 7]. These receptors are normally activated by the transmitter glutamate released from the parallel fibers on distal PC dendrites [8]. In a previous study, we showed that offspring that were prenatally treated with sGCs exhibited a moderate increase in the immunohistochemical expression of PC mGlu1R in both infant (postnatal day 22, P22) and adolescent (P52) rats, but this increase did not reach statistical significance [9]. We believe that this trend may have been non-significant because the histological sample studied was in a small area of the cerebellar vermis (lobule IX). To analyze whether the administration of antenatal sGCs is related to an increase in the cerebellar immunohistochemical expression of mGluR1, we here quantified a more representative sample by analyzing the entire sagittal vermal cerebellar region. Furthermore, to ensure that this potential mGluR1 increase is related to changes in distal PC dendritic growth, we also quantified the number of Golgi-stained PC terminal dendrites in the same cerebellar region and in the same animal from which the immunohistochemically-stained tissue was sampled. Furthermore, to determine whether these PC changes were transient or permanent, we performed histological assessments at three relevant ages: infancy (P22), adolescence (P52) and adulthood (P82).

Materials and Methods

Experimental animals and drug administration

Twelve Sprague–Dawley multiparous rats were used. All animals were housed under the following controlled environmental conditions: temperature (20±1°C), day–night cycle (12 h:12 h, light–dark), with food and water *ad libitum*. Once the animals mated (two females with one male per cage), pregnant rats were placed in individual cages (45×25×20 cm), and gestational day 0 (G0) was determined by the presence of sperm detected in vaginal smears. Pregnant animals were randomly classified into one of two groups: control – saline (CON, n=5) and betamethasone-treated (BET, n=7). The BET mothers were given a single course of betamethasone phosphate subcutaneously (170 μg/kg of body weight, twice, dorsal

neck region) (Cidoten®, Schering-Plough, Inc., Santiago, Chile) on gestational day 20 (G20), separated by an 8-hour interval [10, 11]. The CON mothers were given an equal volume (1 mL) of saline. Twenty-four hours after delivery and until postnatal day 21 (P21), the offspring remained undisturbed in their home cage with the mother (lactation period). At P22, the BET and CON males were weaned and rearranged to 3–4 animals per cage.

All procedures were in accordance with the "Guide for the care and use of laboratory animals" (Institute for Laboratory Animal Research, National Research Council, Washington DC, 2011), and the experimental protocols received approval from the local Animal Ethics Committee.

mGluR1 immunohistochemistry

At P22, P52 or P82, the male animals were deeply anesthetized with isoflurane and pentobarbital, and intracardiac perfusion was performed with 0.9% NaCl followed by 4% paraformaldehyde. Cerebella were removed, post-fixed for one hour, and stored in 30% sucrose at 4°C for seven days (cryoprotection). For the immunohistochemical procedure, one-half of the cerebellar vermis was sectioned at 20 µm with a Thermo Scientific Microm HM525 Cryostat (4–6 sections per rat). Sections that had been previously attached to a slide were washed twice in a phosphate-buffered saline (PBS) for 10 minutes each at 90 rpm and then incubated with 0.5% H_2O_2 (Merck) for 30 minutes, at room temperature. After two additional washes in PBS, the sections were blocked with 3% bovine serum albumin (BSA; Sigma) and 0.4% Triton X-100 (Sigma) for one hour. The primary antibody used was anti-mGluR1 (1/100, rabbit anti-rat: OPA1-04060, Pierce Biotechnology) [12]; the sections were incubated with the primary antibody in blocking solution overnight, at room temperature and under agitation (40 rpm). The tissue was then washed three times with PBS and incubated in 1.5% BSA and 0.2% Triton X-100 for two hours at room temperature with agitation at 40 rpm. The tissue was washed again (three times) with PBS. The secondary antibody used was Biotin-conjugated goat anti-rabbit IgG (H+L) (1/500, 31820, Thermo Fischer Scientific). To visualize the labeled protein, an Avidin–Biotin Peroxidase complex (Vectastain® Elite ABC Kit; Vector Laboratories) was prepared in 1.5% BSA and Triton X-100 and incubated for one hour prior to addition to the substrate, coupled with 3,3'-diaminobenzidine (DAB) for 20 minutes without stirring (ImmPACT DAB Peroxidase Substrate; Vector Laboratories). The sections were finally washed in distilled water for 10 seconds, attached to slides, air-dried, enclosed with Entellan (Merck) and coverslipped. To ensure that the immunostaining coincides architectonically with the cerebellar histology, a Hematoxylin counterstaining was performed (Figure 1; blue color: Hematoxylin staining; brown color: immunopositive mGluR1 staining). The cerebellar vermal sections were coded, and the images were captured with a BioBlue model, BB.1153.PLI, Euromex Microscope. In addition, negative controls (secondary antibody only) were assessed without observing mGluR1-positive staining (not shown). Immunoreactivity was quantified using the Image-J software [*National Institutes of Health* (NIH), Bethesda, MD] using grayscale images (% of controls, arbitrary values). To quantify the immunostaining of mGluR1 in the cerebellar vermis, 10 images per parasagittal section of tissue (10×) were captured per group. Then, using the ImageJ program ("white" color adjusted), images were captured from the vermal-selected areas and the average gray value (sum of the gray values of all pixels divided by the number of pixels) was measured, resulting in a representative optical immunohistochemical emission per each cerebellar vermal section.

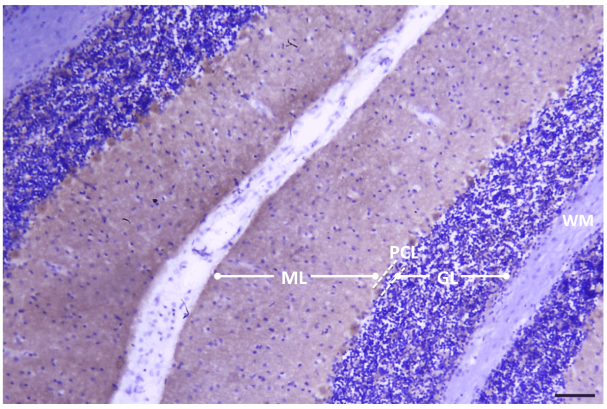

Figure 1 – ***Representative photomicrograph of the cerebellar cortex counterstained with Hematoxylin (blue color) and mGluR1 immunolabeling (brown color). ML: Molecular layer; PCL: Purkinje cell layer; GL: Granular layer; WM: White mater. Dashed parallel lines indicate the location of Purkinje cell somas. Scale bar: 150 µm.***

Purkinje cell dendrites

The other half of the vermal cerebellar tissue was Golgi-stained and sectioned in a sagittal plane. PCs were selected according to previous criteria [13] and then photographed (400×) and digitalized. The procedure was as follows: three images were taken per neuron considering different depths, then were superimposed using the ImageJ program (using the image to stack option followed by "Z projection") obtaining the complete "number of terminal branches per neuron" in a bi-dimensional plane, and counted manually using the Image-Pro Plus software. The number of PCs assessed at each stage was as follows: P22, n=35; P52, n=53; P82, n=53.

Cerebellar and body weights

Each cerebellum was carefully dissected under a stereoscopic lens and freshly weighed (g) on a digital analytical balance (Sartorius–ENTRIS 224). Additionally, body weight (g) was assessed using a digital balance (Radwag–WTB 200).

Statistical analysis

A one-way ANOVA and the Scheffé *post-hoc* test were used to analyze the morphological and immunohistochemical data. The results are presented as the means ± SEM (standard error of the means). The alpha level for the determination of statistical significance was <0.05.

Results

Although infant animals (P22) that were exposed to BET prenatally exhibited no significant change in vermal mGluR1 expression compared with the age-matched CON animals, when animals reached adolescence (P52) or adulthood (P82), the BET-treated animals had a significant

increase in mGluR1 immunolabeling [P52, $F_{(1,4)}$=18.81, **p<0.02; P82, $F_{(1,6)}$=28.97, ***p<0.0001; Figure 2].

Figure 3 shows representative cerebellar vermal micrographs stained with anti-mGluR1 antibody. Immunoreactivity was highly expressed in the molecular layer where dendritic Purkinje cells spanned (asterisks in Figure 3).

Consistent with our previous results, the BET-treated animals exhibited a significant reduction in the number of distal dendritic branches compared with the age-matched controls [P22, $F_{(1,34)}$=6.82, *p<0.05; P52, $F_{(1,52)}$=20.26, **p<0.001; P82, $F_{(1,52)}$=18.87, **p<0.001; Figure 4]. Representative vermal Golgi-stained Purkinje cells from the CON and BET animals are shown in Figure 5.

Compared with the age-matched CON animals, the BET-treated animals exhibited a significant reduction in cerebellar weight at all ages studied [P22, $F_{(1,14)}$=36.8, ***p<0.0001; P52, $F_{(1,14)}$=17.8, **p<0.001; P82, $F_{(1,14)}$=12.5, **p<0.001; Figure 6A]. Moreover, the body weights were reduced at P22 [$F_{(1,14)}$=15.2, **p<0.001] but were normalized in adolescent and young adult animals [P52, $F_{(1,14)}$=3.29, n.s. (not significant); P82, $F_{(1,14)}$=2.64, n.s.; Figure 6B].

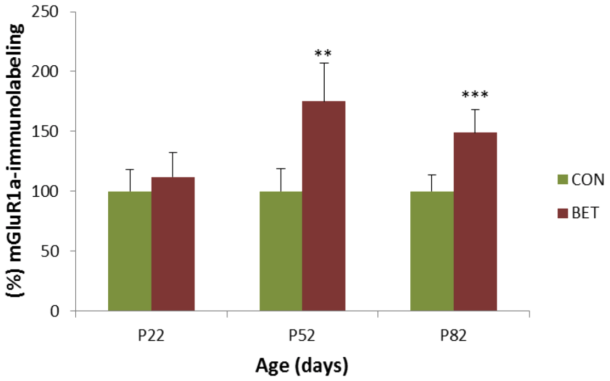

Figure 2 – ***Type-1 metabotropic glutamate receptor (mGluR1) immunoreactivity in the vermal cerebellar molecular layer (expressed as % of control value). CON: Control group; BET: Betamethasone group; P22, P52, P82: Postnatal days 22, 52, and 82, respectively. The data are shown as the means ± SEM (standard error of the means) and are presented as a percentage of control value [P52, $F_{(1,4)}$=18.81, **p<0.02; P82, $F_{(1,6)}$=28.97, ***p<0.0001).***

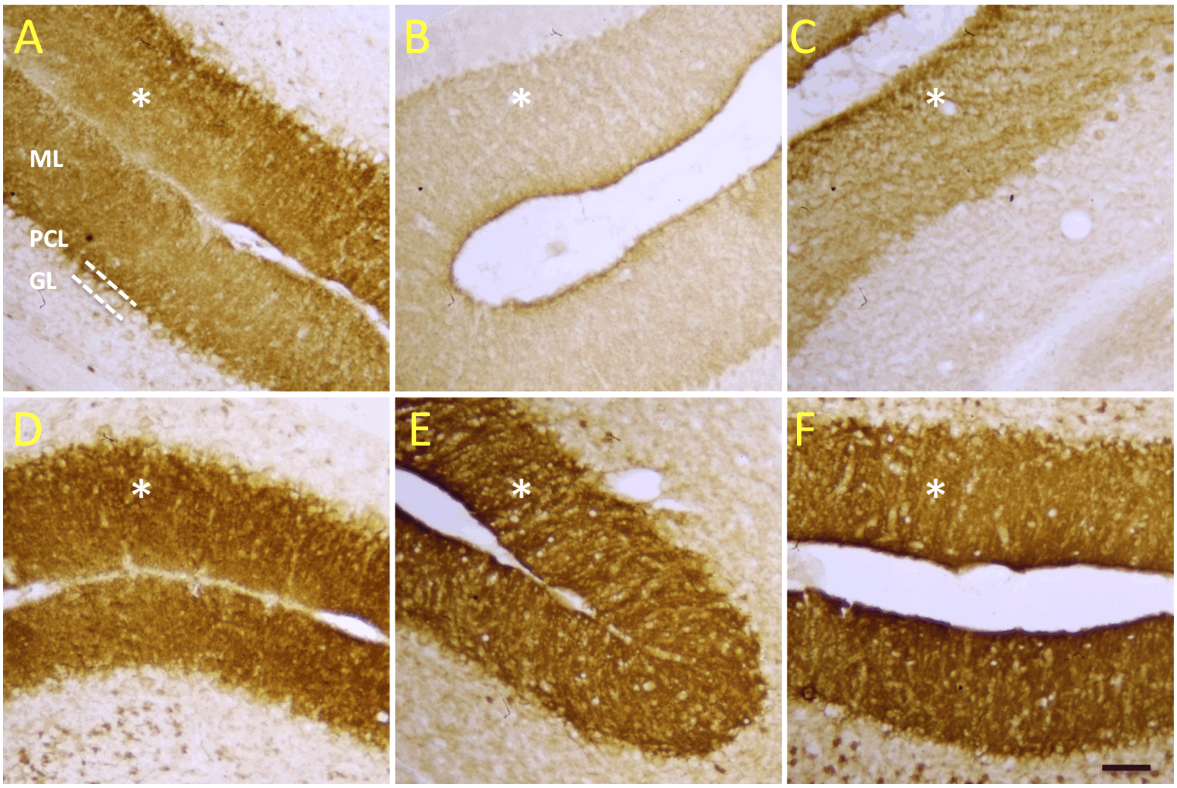

Figure 3 – ***Representative photomicrographs of the cerebellar cortex stained with anti-mGluR1 antibodies from CON (A, B, C) and BET (D, E, F) animals at postnatal days 22 (A, D), 52 (B, E), and 82 (C, F). Asterisks show the cerebellar molecular layer. Scale bar: 100 µm.***

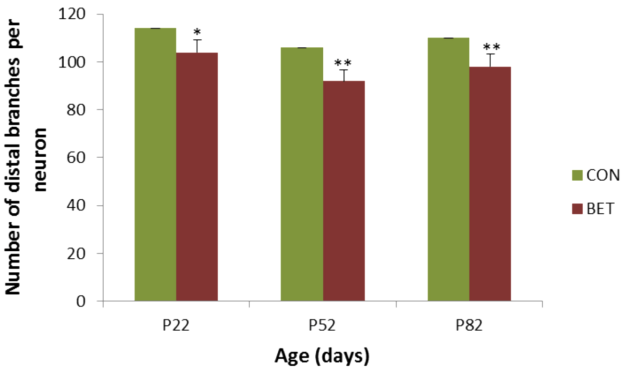

Figure 4 – ***Number of distal dendrites in Golgi-stained vermal Purkinje cells. CON: Control group; BET: Betamethasone group; P22, P52, P82: Postnatal days 22, 52, and 82, respectively. The data are presented as the means ± SEM (standard error of the means) [P22, $F_{(1,34)}$=6.82, *p<0.05; P52, $F_{(1,52)}$=20.26, **p<0.001; P82, $F_{(1,52)}$=18.87, **p<0.001].***

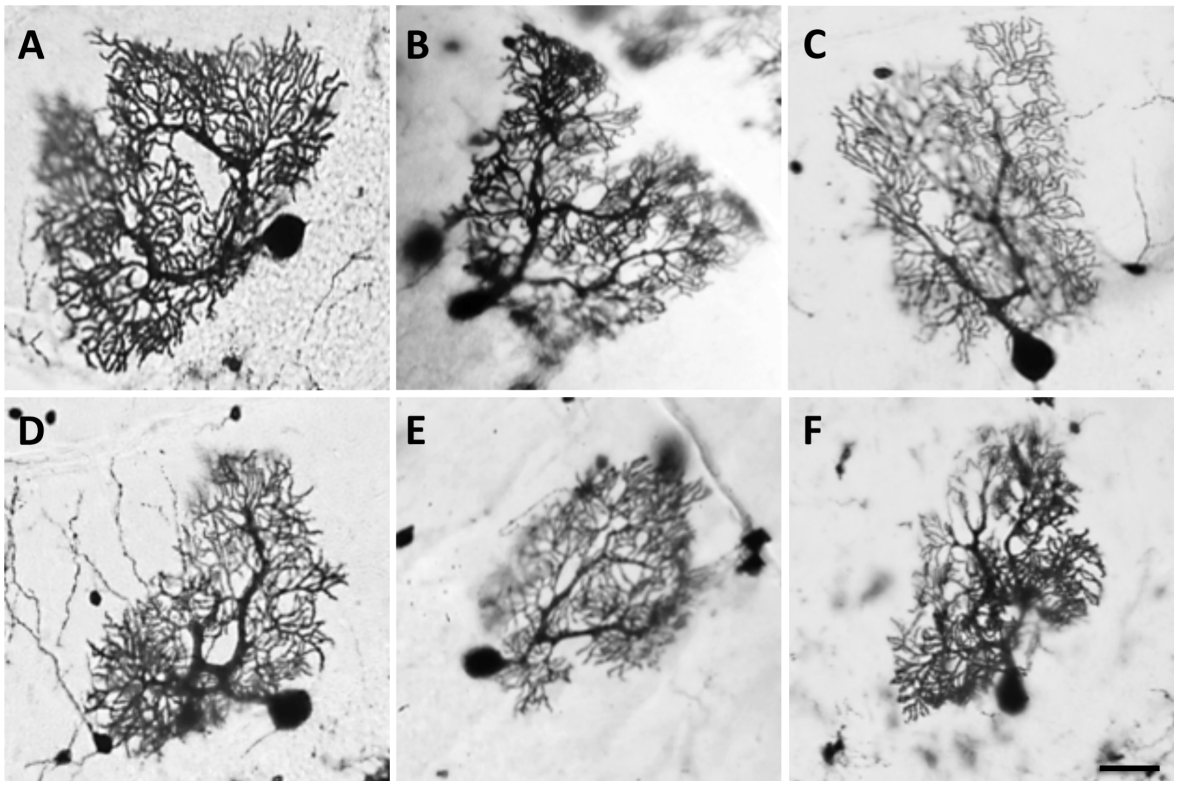

Figure 5 – ***Representative photomicrographs of Golgi-stained vermal Purkinje cells from CON (A–C) and BET (D–F) animals at postnatal days 22 (A and D), 52 (B and E), and 82 (C and F). CON: Control group; BET: Betamethasone group. Scale bar: 40 µm.***

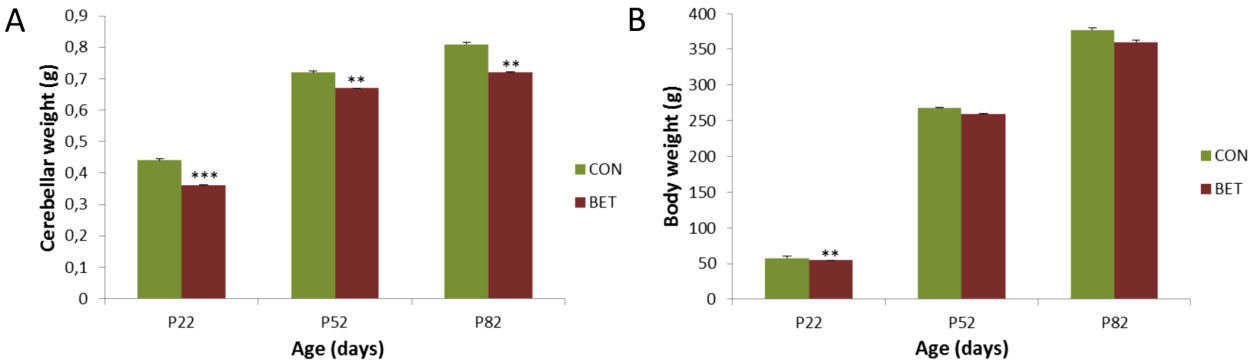

Figure 6 – ***Cerebellar (A; P22, $F_{(1,14)}$=36.8, ***p<0.0001; P52, $F_{(1,14)}$=17.8, **p<0.001; P82, $F_{(1,14)}$=12.5, **p<0.001) and body [B; P22, $F_{(1,14)}$=15.2, **p<0.001; P52, $F_{(1,14)}$=3.29, n.s. (not significant); P82, $F_{(1,14)}$=2.64, n.s.] weights (g) of control – saline (CON) and betamethasone-treated (BET) animals. P22, P52, P82: Postnatal days 22, 52, and 82, respectively. The data are presented as the means ± SEM (standard error of the means).***

Discussion

In the current work, we showed that a single course of prenatal BET administration during the last trimester of gestation (G20) significantly increased the cerebellar immunohistochemical expression of mGluR1 and reduced PC terminal dendrites in cerebellar vermal tissue.

Using a similar paradigm in a very recent study, we were unable to show significant changes in the expression of mGluR1 in BET-treated animals compared with the age-matched CON at both P22 and P52 [9]. The main difference between previous works and the current work is that in our previous study, we evaluated the immunohistochemical expression in a very restricted cerebellar vermal region (lobule IX) and at two ontogenetic stages (P22 and P52); however, in the current study, we assessed the immunohistochemical expression of mGluR1 receptors in all 10 vermal cerebellar lobules (sagittal plane), thus allowing the greater representation of the tissue sample. Additionally, in the present study, we added an additional ontogenetic stage (adulthood, P82) and were thus able to detect a lasting effect of prenatal BET administration on the immunohistochemical expression of mGluR1.

In the cerebellum, the mGluR1s are located primarily in PC dendritic spine annuli. These receptors can be activated by the transmitter glutamate released from the parallel fibers on the distal half of developing PC dendrites, and its endogenous activation is necessary for PC maturation and subsistence. Once activated, mGluR1 generates G-protein dependent intracellular downstream cascades that finally increase the cytosolic free calcium levels [14–16]. If the increased mGluR1 immunohistochemical expression observed in the current study is indirectly related to increased levels of intracellular free calcium,

it is possible to hypothesize that the dendritic deterioration detected in the Golgi-stained neurons is caused by the increased calcium overload, secondary to mGluR1 activity. In fact, we have shown in a previous study that prenatal BET administration significantly increased the immunohistochemical expression of the calcium-sequestering protein calbindin-D28k [2], probably due to free intracellular calcium transients. However, since mGluR1 plays an important role in neuronal plasticity, it is not possible to discard the possibility that the overexpression of mGluR1 would be generated as a compensatory mechanism triggered to minimize the deleterious impact of prenatal BET on PC dendritic development [17, 18].

In this study, we confirmed the effect of prenatal BET exposure on PC dendritic development. By using an animal model of prenatal stress or BET administration, we previously reported a significant reduction in cerebellar PC dendritic area and perimeter per neuron [1, 2]. Although in the current work we quantified a different morphological variable (number of terminal dendritic branches per neuron), all previous and current data are in the same direction, *i.e.*, a prolonged reduction of dendritic material in the rat's vermal PCs. Additional to the above discussed relationship between mGluR1 expression and PC dendritic changes, another possible explanation is that the lower dendrite development induced by prenatal BET administration was due to an indirect, deleterious effect of the cerebellar neural proliferative cells located in the external granular layer of the cerebellum. These late prenatal proliferative cells may give rise to all of the granule cells, whose axons (parallel fibers) constitute the main presynaptic glutamatergic inputs on distal PC dendrites. This conjecture is based on the evidence that the experimental administration of sGCs to weaning mice produces significant increases in the apoptotic proliferative external granular layer cells [19, 20], thus depriving PC dendrites of a large number of inputs. This hypothesis is also supported by studies in which maternal stress significantly alters the synaptic connections between parallel fibers and PC dendrites [14]. Thus, although our current study did not evaluate changes in the number of granule cells, it cannot be ruled out that the lower dendrite development observed in PCs may have been caused, at least in part, by a reduction of presynaptic parallel fibers and may have led to reduced tropic influences on postsynaptic PC dendrites. In fact, we previously show that prenatal BET administration significantly changed the brain-derived neurotropic factor (BDNF) expression in the cerebellar molecular layer of postnatal animals [21].

Nevertheless, our study has several limitations. First, because the mGluR1s are located in the annuli that surround the dendritic spines, it would be valuable to quantify the dendritic spine density per dendritic segment. However, this approach was not possible because the Golgi–Cox technique used in the current study did not stain dendritic spines. Second, the immunohistochemical study is an indirect indicator of the mGluR1 expression, so we cannot determine whether the observed changes in the immunohistochemical expression effectively reflected direct BET-induced mGluR1 protein content. Third, to assess whether prenatal BET administration was responsible for stunted PC dendritic growth, it would be desirable to use *in vitro* GC antagonist agents to demonstrate a direct relationship between sGC administration and PC dendritic development. Future *in situ* and *in vitro* studies are desirable to clarify these points.

Conclusions

Our current data indicate that prenatal BET administration in rats at G20 produced a protracted immunohistochemical overexpression of cerebellar mGluR1 and a significant reduction in distal PC dendritic branching in the offspring. Of note, these and other experimental data do not portend to minimize the beneficial effects of BET administration when there is a risk of respiratory distress/ bronchopulmonary dysplasia in preterm infants.

Conflict of interests

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Author contribution

All authors contributed equally to this work.

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