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Effect of antenatal betamethasone administration on rat cerebellar expression of type 1a metabotropic glutamate receptors (mGluR1a) and anxiety-like behavior in the elevated plus maze

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Summary

Preclinical studies indicate that endogenous or exogenous glucocorticoids acting during the pre- or postnatal periods produce a significant Purkinje cell dendritic atrophy, especially during late postnatal ages. The present authors hypothesized that the underlying substrate that may contribute in part to this morphological change is the under-expression of the metabotropic glutamate 1a receptor (mGluR1a) because its expression is correlated with Purkinje cell dendritic outgrowth. Therefore, in the current study, they analyzed the impact of antenatal betamethasone on the immunoreactive expression of the mGluR1a and on anxiety-like behavior in the elevated plus maze (EPM). Pregnant rats were randomly divided into two experimental groups: control (CONT) and betamethasone-treated (BET). At gestational day 20 (G20), BET rats were subcutaneously injected with a solution of 170 $\mu g.kg^{-1}$ of betamethasone, and CONT animals received a similar volume of saline. At postnatal days 22 (P22) and P52, BET and CONT offspring were evaluated behaviorally in the EPM, and their cerebella were immunohistochemically processed. Contrary to the uthors' expected results, animals that were prenatally treated with a single course of betamethasone did not exhibit under-expression of mGluR1a or behavioral changes consistent with anxiety-like behaviors.

Key words: mGluR1a; Vermal Purkinje cells; Molecular layer; Elevated plus maze.

Introduction

When there is a risk of preterm birth, one of the therapeutic strategies used most frequently is the administration of synthetic glucocorticoids (GCs), i.e., betamethasone or dexamethasone. The rationale of this therapy relies on the fact that these steroidal drugs accelerate the maturation of pulmonary tissue, thus minimizing the risk of respiratory distress and reducing the risk of intraventricular hemorrhage and periventricular leukomalacia [1]. However, the administration of these drugs is not entirely safe for neurological and neuroendocrine development. For example, it has been shown that preterm babies whose mothers were treated with betamethasone showed further alterations in hypothalamic-pituitary-adrenal (HPA) function [2]. Similarly, repeated antenatal betamethasone is associated with higher impulsivity together with fearfulness, tendency to anger, and sadness at two years of age [3].

Furthermore, a recent study has shown that preterm infants whose mothers were treated with synthetic GCs had significantly lower gross cerebellar growth but normal cerebral growth as reported by neuroimaging analysis [4], suggesting that the cerebellar tissue is more vulnerable than previously thought, at least with regards to early treatment with GCs. This increased cerebellar vulnerability is most likely related to the fact that the mammalian cerebellar cortex expresses a remarkable density of glucocorticoid receptors, even exceeding that of the hippocampus or cerebral cortex [5, 6], together with the fact that Purkinje cell dendritogenesis is rapid during the perinatal period [7, 8]. Consistent with these data, in the present authors' laboratory, they have recently observed that the prenatal administration of betamethasone in equivalent therapeutic doses and during a similar ontogenetic stage (gestational day 20) [9, 10] produced a significant alteration in the development of cerebellar Purkinje cells associated with an increase in the calcium sequestering protein calbindin-D28k [11]. It has also been reported that cerebellar Purkinje cell dendritic maturation is related to the expression of type 1 metabotropic glutamate receptors (mGluR1) present in the dendritic membrane. mGluR1 is a family of metabotropic receptors present on various cerebellar cells, including cerebellar Purkinje cells [12], and the membrane density of mGluR1 has been shown to increase concomitantly with dendritic cell outgrowth, in close association with learning phenomena such as long-term

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potentiation [13, 14]. The relevance of these metabotropic receptors for Purkinje cell maturation is evidenced by the fact that in staggerer mutant mice, the abnormal signal between parallel fibers and Purkinje cells is closely associated with alterations in mGluR1 expression and function [15]. Interestingly, the expression of mGluR1 on Purkinje cell dendritic spines is a key developmental process in the normal elimination of exuberant olivo-cerebellar climbing fibers that takes place in dendritic Purkinje cell arborization at gestational day 20 (G20) in rodents [12]. In addition, mGluR1 plays a central role in Purkinje cell plasticity via elevation of free intracellular calcium concentration [16]. On the other hand, GCs appear to interact with mGluRs, and an excess of stress-induced GCs may alter memory formation via changes in the function and expression of mGluRs [17].

Because the direct administration of antenatal synthetic GCs in experimental animals at G20 produces a significant Purkinje cell developmental delay as shown in Golgi-Cox-stained Purkinje cells [11], the present authors hypothesized that one pathophysiological mechanism involved in the impact of GCs on cerebellar maturation could be the under-expression of mGluR1 in the molecular layer where Purkinje cell dendritic tree outgrowth occurs. Furthermore, it should be noted that in rodents [7] as well as in humans [8], Purkinje dendritic maturation occurs during perinatal periods following a progressive outgrowth from the soma (located in the middle cerebellar cortical layer) towards the cerebellar upper molecular layer. Considering those findings, the aim of the current study was to evaluate whether antenatal betamethasone administered at G20 in an equivalent clinical dose (170 $\mu g.kg^{-1}$) [10] alters the postnatal expression of cerebellar mGluR1a in the cerebellar molecular layer. Furthermore, as the main behavioral sequel of antenatal administration of synthetic GCs are anxiety behaviors during childhood and adolescence [18], the authors further assessed whether betamethasone-treated animals exhibit anxiety-like behavior in the elevated-plus maze (EPM).

Materials and Methods

Multiparous female rats were housed under the following controlled environmental conditions: temperature (20 ± 1°C) and day-night cycle (12:12 light-dark), with food and water ad libitum. After mating (one female with one male per cage), females were placed in individual laboratory cages, and the gestational day 0 (G0) was determined by the detection of sperm in vaginal smears. Pregnant rats were randomly classified into the following four experimental groups: (i) saline control animals evaluated at 22 postnatal days (CONT-P22; n = 13), (ii) betamethasone-treated animals evaluated at 22 postnatal days (BET-P22; n = 9), (iii) saline control animals evaluated at 52 postnatal days (CONT-P52; n = 12), and (iv) betamethasone-treated animals evaluated at 52 postnatal days (BET-P52; n = 13). To avoid gender-related influences, the behavioral and neuronal assessments were conducted only in male offspring.

On G20, the mothers of the BET-P22 and BET-P52 groups were given two doses of 170 $\mu g.kg^{-1}$ betamethasone subcutaneously, separated by an eight-hour intervals. The authors used this dose because pharmacokinetic and pharmacodynamics analysis of betamethasone in the rat has found this dose to be equivalent to the 12-mg dose, which is usually administered in two courses separated by an interval of 12 hours in cases of human preterm birth risk [10]. G20 was chosen because it corresponds to the approximate ontogenetic stage of a pre-term human (24-32 weeks gestation) [9, 10]. Control groups received an equal volume (one ml) of saline solution.

All offspring were tested on the EPM at P22 or P52. The EPM was constructed of black wood and consisted of two open arms (60 × 6 cm) and two closed arms (60 × 6 × 14 cm) and was immobile on a fixed base, 41.5 cm above the floor. Each animal was placed in the center of the EPM and allowed to freely explore the maze for five minutes. The following two behavioral variables were evaluated: the percentage of entries in the open arms (% = open arm entries/total entries in all four arms x 100) and the time spent in the open arms (% = time spent in the open arms/ time spent in all four arms x 100). Animals that explore the open arms less frequently and for less time are considered anxious compared to age-matched saline controls animals [19]. Placement of all four limbs into one arm of the maze is defined as an effective entry into that arm. Exploratory behavior in the EPM was recorded using a webcam. Behavioral data were recorded in a blinded fashion and processed with ANY-maze 4.60 software. Behavioral analyses were conducted between 09:00 and 15:00 hours, and the apparatus was carefully cleaned with 5% ethanol between each analysis. The animals were placed in the behavioral assessment room ten minutes prior to behavioral testing to allow habituation to the new environment.

Following behavioral assessment, all male animals (P22 and P52) were deeply anesthetized with isoflurane/pentobarbital, and intracardiac perfusion was performed with 0.9% NaCl followed by 4% paraformaldehyde. Brains were removed, post-fixed for one hour, and stored in 30% sucrose at 4°C for seven days for cryoprotection. Finally, the cerebellar vermis was sectioned at 20 µm for immunohistochemical procedures using a Thermo Scientific Microm HM525 Cryostat, and each cerebellar vermal section was digitalized to measure mGluR1a immunoreactivity in the molecular layer of vermal lobule IX.

Sections that were previously attached to the slide were washed twice in PBS for ten minutes each at 90 rpm and then incubated with 0.5% H_2O_2 for 30 minutes at room temperature. After two additional washes with PBS (1X), the authors proceeded to block with 3% BSA and 0.4% Triton X-100 for one hour. The primary antibody used was anti-mGluR1a (1/1000, ab1778), which was incubated in blocking solution overnight at room temperature under agitation (40 rpms). The tissue was then washed three times with PBS (1X) and incubated in 1.5% BSA and 0.2% Triton X-100 for two hours at room temperature and 40 rpm agitation. The tissue was washed again (three times) with PBS (1X). The secondary antibody, an avidin-biotin peroxidase complex, 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. Finally, the sections were washed with distilled water for ten seconds. The sections were immediately mounted on slides, air-dried, covered with Entellan, and coverslipped. The cerebellar vermal sections were coded and observed using a Motic BA210-Tech Lab microscope. The authors evaluated mGluR1a immunoreactivity (% of controls) using grayscale images with Image-J software (pixel intensity arbitrary values).

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.

The *t*-test was used for the analysis of behavioral and immunohistochemical data. The results are presented as the mean ± SEM. Differences at $p < 0.05$ were considered significant.

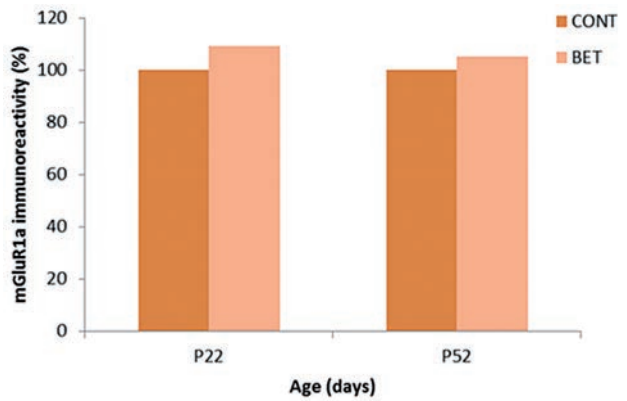

Figure 1. — Immunoreactivity of metabotropic glutamate receptor 1a (mGluR1a) expression in the vermal cerebellar molecular layer. CONT: control group; BET: betamethasone group; P22-P52: postnatal days 22 and 52. Data are shown as the mean ± SEM and are presented as a percentage of the controls (*t*-test).

Results

As shown in Figure 1, animals exposed to betamethasone prenatally on G20 showed no significant change in expression of mGluR1a in either age group studied (P22 or P52), suggesting that betamethasone given at the ontogenetic postnatal stage (G20) and given in a clinically equivalent dose (170 mg.kg^{-1}) did not produce the expected changes. Figure 2 shows four micrographs of tissue sections from the cerebellar vermal zone immunostained with anti-mGluR1a under ×10 magnification. Similar to the immunohistochemical data, betamethasone-treated animals exhibited no behavioral changes compared to saline-control animals (Figure 3). Consistent with previous clinical and preclinical studies, body weight was significantly lower in the betamethasone-treated animals at both P22 and P52 (CONT-P22: 60.1 ± 1.5 g; BET-P22: 53.8 ± 1.1 g; CONT-P52: 265.8 ± 3.1 g; BET-P52: 246.9 ± 2.9 g; p <0.05).

Discussion

In the present study, the authors failed to support the hypothesis that prenatal betamethasone administered in clinically equivalent doses (170 mg.kg^{-1}) alters the immunohistochemical mGluR1a expression in the rat cerebellar cortex. Likewise, animals prenatally treated with betamethasone showed no significant differences in exploratory behavior compared to their age-matched control animals. There are several reasons why the authors were not able to show changes in the expression of mGlu1Rs or in the anxiety-like behavior in the elevated plus maze. First, in this study, immunohistochemical quantification was performed in the molecular layer of the cerebellum, where nearly all dendritic arborization of Purkinje cells occurs; however, because in that layer two other cell types (stellate cells and basket) are found that similarly express mGlul1a receptors, the Purkinje cell immunohistochemical expression of mGluR1a in the molecular layer of animals treated with betamethasone was likely masked by the immunohistochemical expression of the other microneurons residing in this superficial cortical layer. Second, although many mGluRs play a key role in cerebellar development and plasticity [20], in the present study, the authors analyzed the expression of a single subtype of

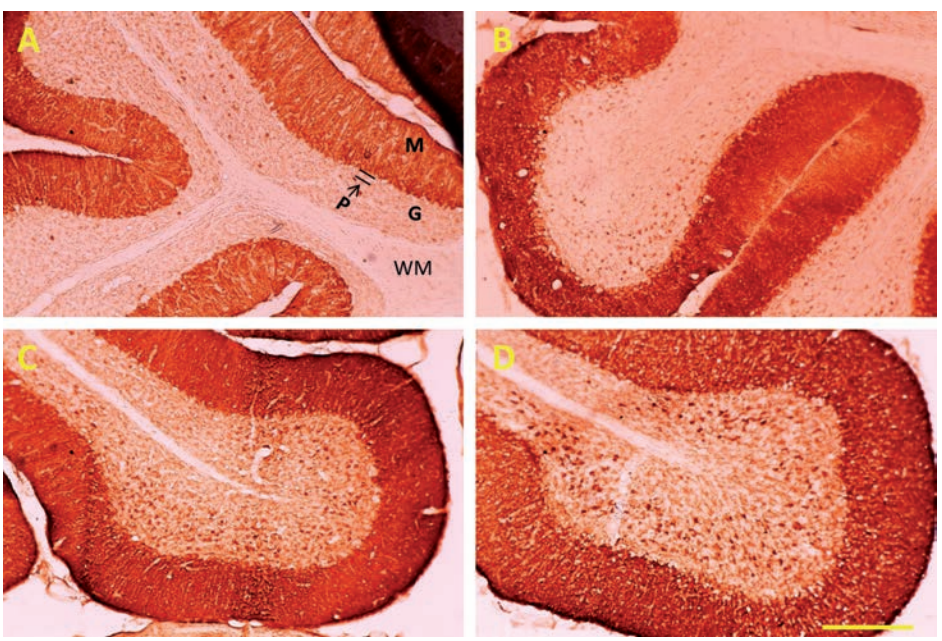

Figure 2. — Representative photomicrographs of vermal tissue sections stained with anti-mGluR1a. A and B: control and betamethasone-treated animals at postnatal day 22; C and D: control and betamethasone-treated animals at postnatal day 52. M: molecular layer; P: Purkinje cell layer (arrow and parallel lines); G: granular layer; WM: white matter. Scale bar: ~300 µm.

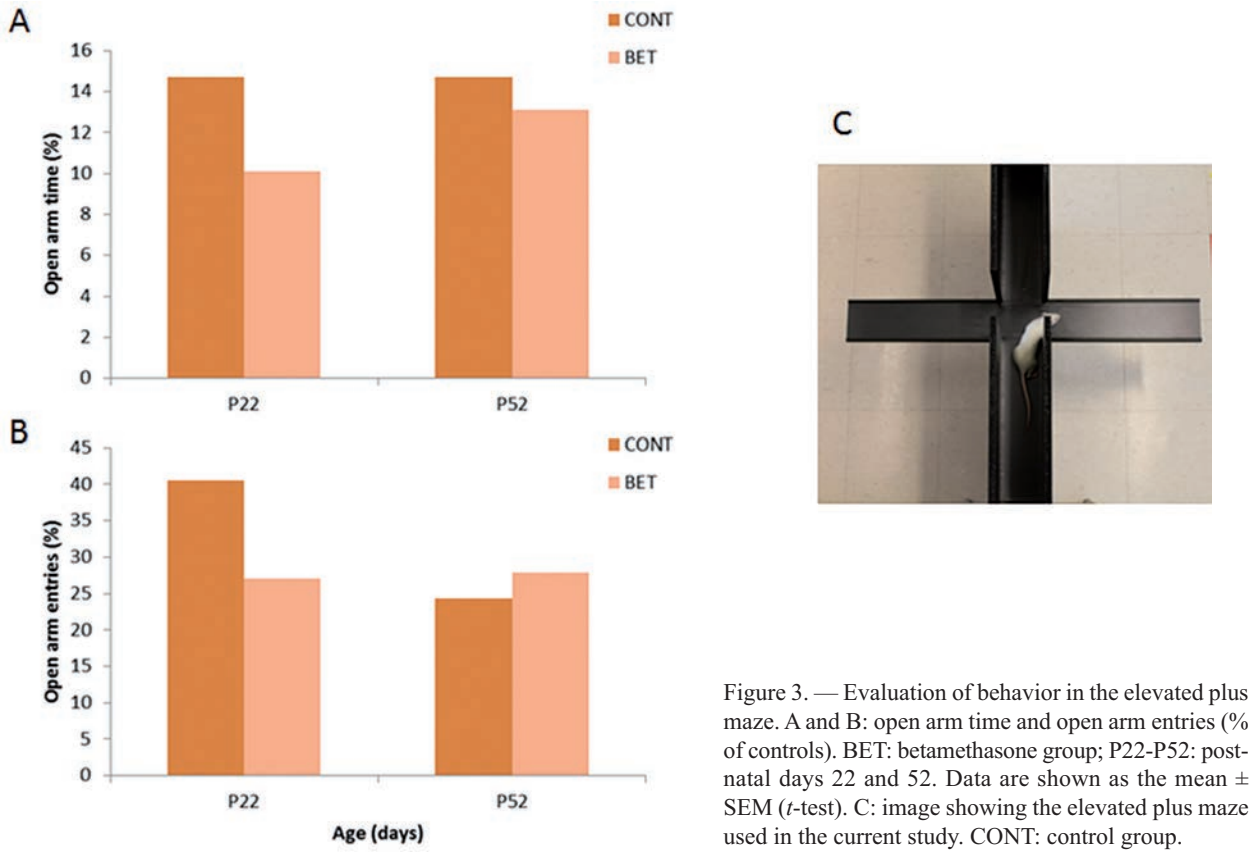

Figure 3. — Evaluation of behavior in the elevated plus maze. A and B: open arm time and open arm entries (% of controls). BET: betamethasone group; P22-P52: postnatal days 22 and 52. Data are shown as the mean ± SEM (*t*-test). C: image showing the elevated plus maze used in the current study. CONT: control group.

mGluR1 (mGluR1a), but there are other splice variants (mGluR1b-g) that may or may not have been affected by prenatal betamethasone administration and may be responsible for masking the immunoreactivity expression of mGluRs. Third, in order to perform reliable comparisons between the control and betamethasone-treated groups, the authors evaluated the immunohistochemistry expression in the vermal cerebellar lobule IX but not in other vermal lobules that could exhibit different degrees of vulnerability; therefore, it is not possible to know if there were changes in other regions of the cerebellar cortex. Fourth, the ontogenetic stage at which the animals were evaluated (P22 and P52) may have influenced the present results. For example, in the authors' laboratory, rats prenatally treated with a similar dose of betamethasone did not show changes in MAP-2 immunostaining evaluated at P22 and P52; however, Bruschettini *et al.* [21] showed that MAP-2 immunoreactivity was decreased in rats subjected to a single course of prenatal betamethasone with MAP-2 changes detected at P150. Future long-term studies could clarify this issue.

The lack of significant behavioral differences in the EPM between control animals and those prenatally treated with betamethasone was unexpected. The EPM is a test designed and validated to assess anxiety-like behaviors in rodents [22]. In the present authors' previous study [11] using another less-specific exploratory device (an open-field test), animals treated with betamethasone explored the arena significantly less compared to their age-matched controls. Because the lower exploratory activity of animals in this previous study occurred in the center of the open field, they interpret this behavior as an anxious state with avoidance of the ethologically "risky" central zone. However, considering the results of the current study and the higher specificity of the anxiety test that was used (EPM), it seems likely that the lower exploratory behavior in the open field in the previous study could be due to subtle motor alterations rather than anxious behavior. To clarify this issue, future studies are needed to see if a more specific motor test assessment, for example, the rotarod test versus the elevated plus maze, can determine whether the potential behavioral disorders in betamethasone-treated animals are motor or emotional in nature, assuming that these behaviors do in fact exist.

The present authors' interest in the medial cerebellar zone is based on the fact that Purkinje vermal cells, by exerting inhibitory influences on the cerebellar fastigial nucleus, can modulate affective states and, during perinatal development, can exert powerful influences on neurobiological processes involved in the wiring of cerebello-limbic connections. In fact, it has been demonstrated that the vermal cerebellar region is directly and indirectly connected with the hypothal-

amus, hippocampus, amygdala, nucleus accumbens (ventral striatum), and cingulate cortex, all of which are involved in the modulation of affective traits or states [23]. Interestingly, Anderson *et al.* [24] showed that the cerebellar vermis but not the cerebellar hemispheres of adults sexually abused in childhood exhibits significant alterations according to functional magnetic resonance imaging (fMRI) (T2 relaxometry), confirming the vulnerability of the vermal cerebellar region to early stressful experiences. Of note, early stressful experiences produce a significant and sometimes permanent alteration in the function of the HPA axis that releases excessive cortisol in response to normal stressful experiences during postnatal life [25].

In summary, in the current study, the authors showed that a single course of betamethasone, in a clinically equivalent dose and during a prenatal stage similar to the antenatal period during which a human fetus is habitually exposed, had no effect on mGluR1a expression in the vermal cerebellar molecular layer or on the exploratory behavior in the EPM. Furthermore, because the authors studied two ontogeny stages (P22 and P52), it is not possible to rule out the existence of neurobehavioral changes before the postweaning period or after postnatal day 52, as has been shown in previous studies using the paradigm of prenatal stress [11].

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