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Original Article

Influence of antenatal synthetic glucocorticoid administration on pyramidal cell morphology and microtubule-associated protein type 2 (MAP2) in rat cerebrocortical neurons

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Abstract. Previous animal studies have indicated that excessive prenatal circulating glucocorticoid (GC) levels induced by the antenatal administration of synthetic GC (sGC) significantly alter neuronal development in the cerebellar and hippocampal neurons of the offspring. However, it is unknown whether antenatal sGC administration results in long-term neocortical pyramidal cell impairment. In the current study, we examined whether an equivalent therapeutic dose of antenatal betamethasone phosphate (BET) in pregnant rats alters the Golgi-stained basilar dendritic length and histochemical expression of dendritic microtubule-associated protein 2 (MAP2) of neocortical pyramidal cells in infant, adolescent, and young adult offspring. The results obtained showed that *in utero* BET exposure resulted in a significant reduction in the basilar dendritic length per neuron and a transient reduction in histochemical MAP2 immunoreactivity. Consistent with previous hippocampal and cerebellar data, the present findings suggest that prenatal BET administration alters the dendritic growth of cerebrocortical pyramidal cells.

Key words: pyramidal cells, glucocorticoids, microtubule-associated protein 2 (MAP2), dendritic outgrowth

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Introduction

Abnormal amounts of maternal circulating glucocorticoids (GCs), which can be induced by conditions such as chronic maternal stress, may produce protracted neurobehavioral alterations in the offspring. It has been shown that maternal prenatal (but not postnatal) emotional stress is linked to abnormal infant affective reactivity at 4 mo of age (1) and reductions in gray matter density in various brain regions, including the prefrontal, premotor, temporal, and cerebellar cortices, at 6–9 yr of age (2). Similar results

have been reported in animal models of prenatal stress, with offspring showing long-term anxiety-like behaviors and delayed dendritic morphology in prefrontal (3), hippocampal (4), and cerebellar Purkinje cells (5). Because stressful conditions produce elevated levels of circulating GCs, neurobehavioral impairments have been attributed to the toxic effects of excessive circulating prenatal GCs (6–8).

In our laboratory, we have systematically studied the effect of controlled prenatal synthetic GC (sGC) administration (betamethasone, BET) to the mother on behavioral and neuronal development in the offspring. We have observed that administering BET to pregnant rats at a therapeutic dose equivalent to that administered to women who are at risk of preterm delivery produces a significant decrease in dendritic arborization in both dentate granule and cerebellar Purkinje cells (9, 10). However, it is unknown whether similar neuronal changes to pyramidal cortical cells occur during the early and late postnatal periods. Thus, in the current study, we analyzed the impact of prenatal BET administration (0.17 mg/kg) (11–13) on the dendritic growth of layer II/III pyramidal cells using the Golgi-stained procedure along with histochemical staining of the dendritic marker microtubule-associated protein 2 (MAP2) in infant (postnatal day 22, P22), adolescent (P52), and young adult (P82) rats.

Subjects and Methods

Experimental animals and drug administration

Eleven pregnant multiparous Sprague-Dawley rats were housed under controlled environmental conditions (temperature, 20 ± 1 C; 12h:12h light-dark cycle) with food and water available *ad libitum*. Pregnant animals were placed in individual cages (45 cm × 25 cm × 20 cm), and gestational day 0 (G0) was determined by the presence of sperm detected in vaginal smears. Rats were randomly classified into the following two groups: control-saline (CON, n = 6) and betamethasone-treated (BET, n = 5). BET-treated rats were given a single course of betamethasone phosphate subcutaneously (0.17 mg/kg of body weight in the dorsal neck region; Cidoten®, Schering-Plough, Inc., Santiago, Chile) on gestational day 20 (G20), with the doses separated by an 8-hour interval. Importantly, this rodent developmental stage corresponds approximately to a human fetus at 28–36 gestational wk (14). Furthermore, according to the rat's BET pharmacokinetics and pharmacodynamics, the dose used in the current and previous studies was equivalent to that administered to a woman who is at risk of preterm delivery (11, 13). After the pre-weaning period (postnatal day 22, P22), the CON (n: 23) and BET (n: 28) males were weaned and rearranged to house 2–3 animals per cage.

All procedures involving animals were approved by the local animal ethics committee and 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).

Dendritic branching

All male animals were deeply anesthetized with pentobarbital (50 mg/kg of body weight; Sigma-Aldrich, Co., 3050 Saint Louis, Misuri, USA), and intracardiac perfusion was performed with 0.9% NaCl followed by 4% paraformaldehyde (Sigma-Aldrich). Each brain was carefully dissected under a stereoscopic lens and freshly weighed (g) on a digital analytical balance (Sartorious, ENTRIES 224). Additionally, body weight (g) was assessed using a classical balance (Radwag-WTB200). Parietal slices were Golgi-stained (see 15 for further details), stabilized with collodion solution (Fluka, Sigma-Aldrich), and sliced into 120-µm thick sections. Pyramidal cells were selected according to the following criteria: (1) having a well-defined pyramidal shape, (2) having a single apical dendrite oriented perpendicularly to the pial surface, (3)

demonstrating an adequate staining of the soma and dendrites, (4) having no extensive processes overlapping neighboring neurons, and (5) located in a cortical strip at 350–600 µm under the pial surface (cortical layers II/III were delimited by the stereotaxic coordinates as described in Paxinos and Watson, 1998) (16). Selected neurons were then photographed (400 ×) and digitized, and the basilar dendritic length (µm) per neuron was quantified. The number of pyramidal cells assessed at each stage was as follows: CON-P22: 178; BET-P22: 135; CON-P52: 120; BET-P52: 160; CON-P82: 160; and BET-P82: 160.

MAP2 immunohistochemistry

The contralateral hemisphere of each animal was post-fixed for one hour and stored in 30% sucrose at 4 C for 7 d (cryoprotection). For the immunohistochemical procedure, the parietal zone was sliced into 20-µm-thick sections with a Cryostat Thermo Scientific Microm HM525 (Walldorf Baden-Wurtemberg, Germany), 4–6 sections per rat. Sections that had been previously attached to a coded slide were washed twice in phosphate-buffered saline (PBS) for 10 min per wash at 90 rpm and then incubated with 0.5% H_2O_2 (Merck) for 30 min at room temperature. After two additional washes in PBS, the sections were blocked for 1 h with 3% bovine serum albumin (BSA; Sigma-Aldrich) and 0.4% Triton X-100 (Sigma-Aldrich). The primary antibody used was monoclonal anti-MAP2 (M4403, Sigma-Aldrich). 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 2 h at room temperature and under agitation (40 rpm). The tissue was washed again (three times) with PBS. The secondary antibody used was conjugated goat anti-affine pure rabbit IgG (H + L) for MAP2 incubation (1:500) diluted in 1.5% BSA and Triton X-100 at 0.2% for 2 h at room temperature, without agitation. To visualize the labeled parietal MAP2 expression, 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 diaminobenzidine (DAB) for 20 min without stirring (ImmPACT DAB Peroxidase Substrate; Vector Laboratories). The sections were finally washed in distilled water for 10 sec, attached to coded slides, air-dried, enclosed with Entellan (Merck), and coverslipped. Images from the parietal cortex coronal sections were captured with a BioBlue model, BB.1153. PLI, Euromex Microscope, considering the same cortical region used in the Golgi-stained cortical tissue (located at 350–600 µm under the pial surface, corresponding to approximately layers II/III). MAP2 immunoreactivity was evaluated with ImageJ software (NIH, Bethesda, MD, USA) using grayscale images (% of controls, arbitrary values). The number of brain sections assessed per animal was as follows: CON-P22, n: 4; BET-P22, n: 3; CON-P52, n: 4; BET-P52, n: 4; CON-P82, n: 3; and BET-P82, n: 3.

Statistical analysis

A one-way analysis of variance (ANOVA) and Scheffé post-hoc test were used to analyze the basal dendritic length per neuron. For immunohistochemical data, we used the Kruskal-Wallis (KW) non-parametric test. The results are presented as mean ± SEM. The alpha level for the determination of statistical significance was < 0.05.

Results

Golgi-stained layer II/III pyramidal cell basal dendrites located in the parietal cortex of animals treated prenatally with BET showed a significant reduction in length compared with the age-matched CON animals at both P52 and P82 (Fig. 1A; **$p<0.01$, ***$p<0.001$, ANOVA). Fig. 1B shows representative layer II/III pyramidal cells from each condition and age.

However, animals exposed to prenatal

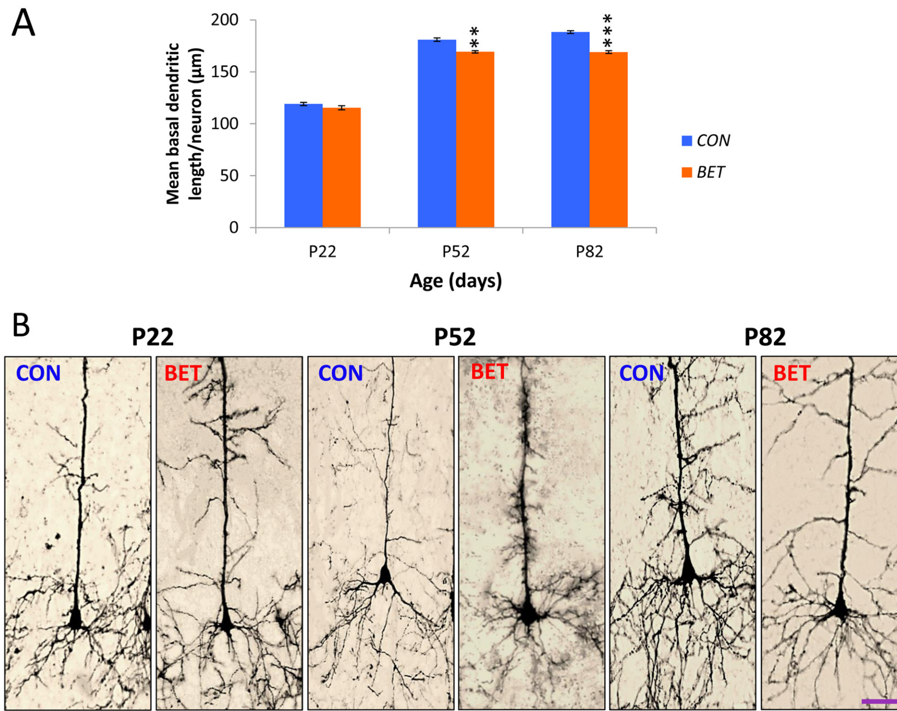

Fig. 1. (A) Mean basilar dendritic length per neuron in Golgi-stained superficial (layer II/III) pyramidal cells. CON: control group, BET: betamethasone group. P22, P52, P82: postnatal days 22, 52, and 82, respectively. The data are presented as the mean ± SEM (**$p<0.01$, ***$p<0.001$, one-way analysis of variance). (B) Representative photomicrographs of Golgi-stained pyramidal cells (layers II/III) from CON and BET animals. Bar: 40 µm.

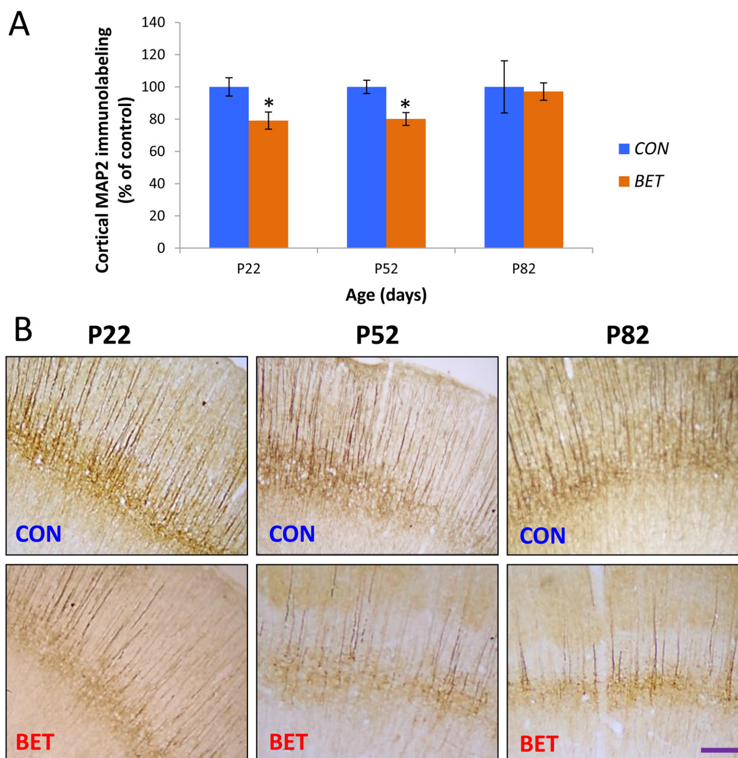

Fig. 2. (A) MAP2 immunoreactivity in layer II/III parietal cortex. CON: control group; BET: betamethasone group; P22, P52, P82: postnatal days 22, 52, and 82, respectively. The data are shown as mean ± SEM and are presented as a percentage of the control value (*$p<0.05$, Kruskal-Wallis). (B) Representative photomicrographs of the parietal cortical tissue stained with anti-MAP2 antibodies from CON and BET animals. Bar: 120 µm.

BET showed a significant reduction in MAP2 immunohistochemical expression at both P22 and P52 but not at later ages (P82) (Fig. 2A) (*$p<0.05$, KW). Fig. 2B shows representative neocortical (parietal) micrographs from CON and BET offspring with the immunohistochemical MAP2 labeling of superficial (layers II/III) pyramidal cells at P22, P52, and P82.

Discussion

In the current study, we showed that a single course of prenatal BET causes a reduction in basilar dendritic length per neuron in layer II/III neocortical pyramidal cells and a transient reduction in neocortical histochemical MAP2 immunoreactivity.

Consistent with our previous studies in the rat cerebellar cortex, adolescent (P52) and young adult (P82) animals exposed prenatally to a single course of BET showed a significant reduction in basal dendritic trees in the superficial neocortical pyramidal neurons (layers II/III). The long-term morphological alteration of dendritic development is the most consistent result observed in our studies. For example, dentate granule cells of adolescent rats (P52) treated with prenatal BET (G20; 0.17 mg/kg) showed a significant reduction (–35%) in total dendritic length compared with age-matched controls (9). A similar reduction in dendritic domain (–39%) has been observed in cerebellar Purkinje cells at P52 in offspring treated antenatally with BET (10). In both studies, the neuronal changes were related to significant impairments in spatial memory tasks along with anxiety-like behaviors. Although we did not evaluate functional variables, it is possible that the reduced dendritic arborization could alter pyramidal cell function because the majority of synaptic connections, transmission, and signal integration significantly depend on the number, length, and complexity of the cell's dendritic branching (17). Moreover, since the glucocorticoid receptor (GR) is highly expressed in layers II/III cerebrocortical neocortical neurons (18), the morphological changes in pyramidal cells reported here are probably due to long-term effects of prenatal sGC administration on cortical neuronal maturation. The results of the current study in conjunction with previous studies on dendritic morphometry indicate that prenatal sGC administration could produce subtle microstructural changes that may be related to the long-term behavioral and cortical findings described in children whose mothers experienced stressful prenatal conditions (1, 2).

Moreover, MAP2 is the most predominant cytoskeletal protein isoform present in dendritic branches; it contributes to dendritic growth and plasticity (19, 20). Regarding the results obtained in the present study, although we observed that the reduction of MAP2 in the neocortex of animals exposed prenatally to BET is protracted (infant and adolescent rats), we did not observe significant differences when the animals reached young adulthood (P82). This transient reduction in MAP2 is consistent with our observation in previous studies carried out in cerebellar neurons at the same ontogenetic age (P52, –56%) (12). It should be noted that the mere transience of the reduction in immunohistochemical expression of MAP2 does not rule out the possibility that MAP2 underexpression changes the course of neuronal dendritic maturation and causes permanent structural dendritic developmental impairments, as observed in the current Golgi-stained layer II/III pyramidal cells. However, because immunohistochemical MAP2 staining is an indirect measure of protein expression, it is difficult to determine whether the transient MAP2 reduction observed in BET-treated animals clearly reflects quantitative changes in MAP2 protein expression. To make this differentiation, it is necessary to complement the immunohistochemical approach used in the current work with other quantitative methods such as western blot analysis. In addition, since our previous studies carried out in the cerebellar cortex indicated significant changes in the immunohistochemical expression of BDNF

and its TrkB receptor, along with MAP2, in the Purkinje cells of animals exposed to prenatal BET, it is not possible to rule out that the changes observed in the present study also involve changes in these neurotrophic variables.

In conclusion, the present data indicate that administration of prenatal BET to pregnant dams at G20 is associated with a long-term reduction in the basilar dendritic length of cortical pyramidal cells and a transient reduction of the immunohistochemical expression of MAP2 in superficial II/III neocortical cells of the offspring.

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