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Rat psychomotor development and apoptotic protein expression in their brains after glucocorticoid treatment

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Abstract. A single injection of low-dose dexamethasone on postnatal day 3 resulted in the upregulation of key apoptotic enzyme – active caspase-3 in the rat cortex 120 hours after injection, and was followed by a delayed development of neonatal startle reflex and cliff avoidance reaction, an arrest of body weight gain and reduced spontaneous locomotor activity of pups. A single hydrocortisone administration to neonatal rats was only followed by a short-term delay in body weight gain, with no changes in the levels of active caspase-3 in the cortex and brainstem, as well as with no abnormalities in neurodevelopment. These results evidence for lesser neurotoxicity of natural hormone during neonatal development in comparison with dexamethasone and suggest the possibility that hydrocortisone might be used as a substitution for its synthetic analogue in the perinatal medicine.
Keywords: Brain, neurodevelopment, glucocorticoids, apoptosis, rat development.

Introduction

Synthetic glucocorticoid dexamethasone improves perinatal lung maturation and is widely used to prevent the respiratory distress syndrome in neonates (Jobe 2009). However, both clinical studies and laboratory animal researchs indicate that perinatal dexamethasone treatment can have deleterious effects on central nervous system and behavioral development (Watterberg 2007; Noguchi *et al*. 2008; Jobe 2009). The damaging effects of dexamethasone may be related to the attenuation or augmentation of apoptosis – the naturally occurring cell death (Noguchi *et al*. 2008), which is much more active in the developing than in the adult mammalian brain (White & Barone 2001, Menshanov *et al*. 2006, Men'shanov *et al*. 2011).

There are two possible ways to reduce the dexamethasone-related developmental neurotoxicity: 1) lowering the dosage of drug or 2) using a different steroid. However, the molecular and behavioral consequences of the administration of a single low-dose dexamethasone or hydrocortisone to neonatal rats are poorly understood. According to this, the goal of the present research was to study the effects of low-dose dexamethasone and hydrocortisone on general and psychomotor development of neonatal rats and the expression of the key apoptotic enzyme – procaspase-3 and its active form in the brain regions with high intensity of developmental apoptosis – brainstem and cortex.

Materials and Methods

Animals and treatment

All animal procedures were in compliance with the European Communities Council Directive of 22.09.2010 (EC 2010/63/EU) and were approved by the institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable data. Wistar rat pups raised in the colony of our institute were used in experiments. The colony was maintained under natural illumination at 22-24ºC with food and water available ad libitum. The day of birth was considered as postnatal day 1 (PD1). Litters were culled to 8 pups on PD3. On PD8 animals were treated subcutaneously either with 5 mg/kg of hydrocortisone or 0.2 mg/kg of dexamethasone in 20 ul of saline. Control animals received an equal volume of saline. All rats were divided into two experimental groups. Animals of the first group were used for behavioral testing. Rats of the second group were used for biochemical determinations and were rapidly decapitated in 6, 24 or 120 hours after injection. Tissue samples – brainstem and cortex - were dissected out on a cooled plate and frozen in liquid nitrogen until further processing.

Behavior testing

To determine the effect of glucocorticoid administration on the postnatal psychomotor development of mice, all animals of the first group were assessed for behavioral changes with

tests included in the Fox's battery for rats (Fox 1965). All testing was performed between 10:00 and 14:00 from PD4 till PD18. Moreover, body weight gain was determined each day after glucocorticoid administration.

Righting reflex and Cliff drop aversion

The animal was placed face up and the time taken to turn over to a prone position with all four feet on the floor was assessed. The maximum time allocated to perform the test was 60 seconds. The pup was placed on the edge of the table with the forepaws and head extending over the edge. The response was scored as positive if the rat turned and crawled away at least 90º from the "cliff". The maximum time allocated to perform this test was 60 seconds.

Negative geotaxis and Immunoblot analysis

The animal was placed facing downwards on a 30º incline and the latency to turn 180º was recorded. The maximum time allocated to perform the test was 30 seconds.

Spontaneous locomotor activity. The detection of locomotor activity was carried out in a warm (30°C) plastic chamber (30x20 cm) for 60 sec as described previously by Menshanov & Dygalo (2007). Brain tissue was homogenized in lysis buffer containing 150 mM NaCl, 50 mM Tris, 1% Triton X-100 and following protease inhibitors: 1 mM phenylmethylsulfonylfluoride and 2 μg/ml of leupeptin, pepstatin and aprotinin. Electrophoresis was used to separate aliquots (50 ug) of a total protein on 15% sodium dodecyl sulfate polyacrylamide gel. The resolved proteins were transferred on the nitrocellulose membrane by Transblot Cell (Bio-Rad Laboratories, USA). Ponceau S staining was used to control equal loading of the samples and protein transfer to membrane. Detection of caspase-3 forms was performed as it was described previously (Menshanov *et al*. 2006) with polyclonal primary rabbit antibodies (1:250 for intact and active caspase-3; 1:1000 for actin) and secondary alkaline phosphatase conjugated goat anti-rabbit antibody (1:500). Intensities of the signals for procaspase-3 and active caspase-3 bands were in a range of a linear dependence on these proteins amounts.

Results and Discussion

Dexamethasone administration on PD3 resulted in the arrest of body weight gain during the first 24 hours after injection. The weight increase was restored only 48 hours after injection, leading to a significant difference between dexamethasone-treated and control animals (Fig. 1; $F_{(30,420)}=2.000$, $p<0.0017$). The administration of the equivalent dose of hydrocortisone was less detrimental, and animals recovered the body weight gain in 24 hours after injection (Fig. 1). The observed changes were the classic hallmark of glucocorticoid inhibitory action upon the metabolism of developing rats (He *et al*. 2004). Such catabolic action of corticosteroids is often followed by the activation of the key apoptotic enzyme – caspase-3 in different periferal tissues (Viegas *et al*. 2004).

However, neither dexamethasone nor hydrocortisone did not affect the expression of active caspase-3 in the brainstem and cortex of treated rats in 6 or 24 hours after injection (Fig. 2A-B; Cortex +6 hours - $F_{(2,15)}=0.315$, $p=0.734$; Brainstem +6 hours – $F_{(2,13)}=0.166$, $p=0.849$; Cortex +24 hours - $F_{(2,17)}=1.564$, $p=0.238$; Brainstem +24 hours – $F_{(2,17)}=0.673$, $p=0.523$). As the activation of caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis (Jänicke *et al*. 1998), the single administration of the low dose of glucocorticoids did not induce immediate upregulation of apoptosis, as it was shown earlier for higher doses in other brain regions (Noguchi *et al*. 2008).

The level of procaspase-3 was also stable 6 and 24 hours after administration of dexamethasone or hydrocortisone (Fig. 2C-D; Cortex +6 hours - $F_{(2,15)}=0.003$, $p=0.997$; Brainstem +6 hours – $F_{(2,15)}=0.132$, $p=0.878$; Cortex +24 hours - $F_{(2,17)}=0.050$, $p=0.952$; Brainstem +24 hours – $F_{(2,17)}=0.486$, $p=0.623$). The lack of immediate changes in the levels of caspase-3 proenzyme after corticosteroid injection evidenced that transcription of *caspase-3* gene was not controlled directly by glucocorticoids neither in the brainstem nor in the cortex of developing rats.

Nevertheless, a number of factors, such as brain mechanic injury, hypoxia and/or ischemia, are able to influence the intensity of apoptosis in the developing CNS not only in the first few hours after injection, but also in a later life (Bannova *et al*. 2004; Nakajima *et al*. 2000). Such delayed activation of apoptosis might be as deleterious as the immediate one, and could lead to brain damage during development (Nakajima *et al*. 2000). The analysis of active

caspase-3 levels revealed the increased levels of this enzyme in the cortex of rats received dexamethasone 120 hours after injection (Fig. 2A-B; $F_{(2,17)}$=5.362, p=0.016), similar to those observed earlier (Men'shanov *et al*. 2012). However, we did not find any significant upregulation of this apoptotic enzyme after administration of hydrocortisone. The observed difference in the action of these two glucocorticoids in the developing cortex could not be accounted for by the difference in the glucocorticoid receptor affinity for these ligands, as the equivalent dose of drugs was used in our experiment (Rivkees 2008). Developing cortex also lacked the expression of another hydrocortisone molecular target – mineralocorticoid receptors, and hydrocortisone should act in this tissue in the same way as its synthetic analogue (Czock *et al*. 2005). However, natural glucocorticoid could be inactivated by the 11β-HSD2 enzyme, which is expressed in this brain region during neonatal period, and its action might explain the observed difference (Robson *et al*. 1998).

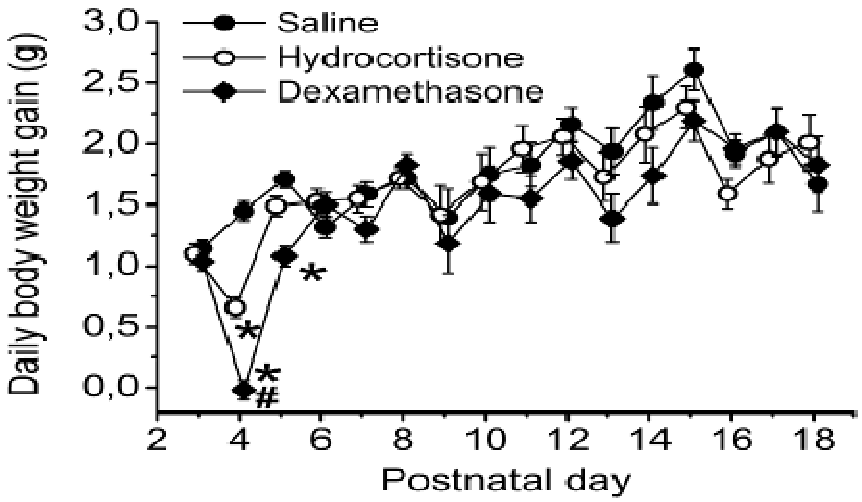

Figure 1. Day-to-day body weight gain after injection of hydrocortisone or dexamethasone on PD3. * - p<0.05 vs saline-treated animals of the same age. # - p<0.05 vs hydrocortisone-treated animals of the same age.

There were no significant changes in the levels of active caspase-3 in the rat brainstem 120 hours after injection (Fig. 2A-B; $F_{(2,15)}$=0.447, p=0.648). The levels of procaspase-3 were also stable in both studied brain regions within this time point (Fig. 2A-B; Cortex - $F_{(2,17)}$=0.314, p=0.735; Brainstem - $F_{(2,17)}$=1.436, p=0.265). We also investigated whether dexamethasone-mediated upregulation of active caspase-3 observed in the cortex was associated with the changes in the psychomotor development of our animals. The administration of synthetic glucocorticoid was followed by a number of developmental changes – there was a delay in the development of cliff avoidance reaction (Fig. 3; $F_{(2,37)}$=2,981, p<0,063) and neonatal startle reflex (Fig. 4A; $F_{(8,112)}$=4,620, p<0,00007), as well as a speed-up of the eyelid opening (Fig. 4B; $F_{(8,112)}$=6,904, p<0,00001). Animals received dexamethasone were also less active than control ones ($F_{(2,26)}$=16,515, p<0,00002). However, the injection of equivalent dose of hydrocortisone was followed only by a speed-up of the eyelid opening (Fig. 3-4), and no behavioral changes were observed in the rats received this drug.

It also should be noted that there were no any developmental changes in first few days after injection of synthetic corticosteroid, and dexamethasone-induced behavioral changes coincided and might be linked with the caspase-3 activation pattern in the cortex by this drug. Thus, low-dose dexamethasone is more toxic than the low-dose hydrocortisone during neonatal development.

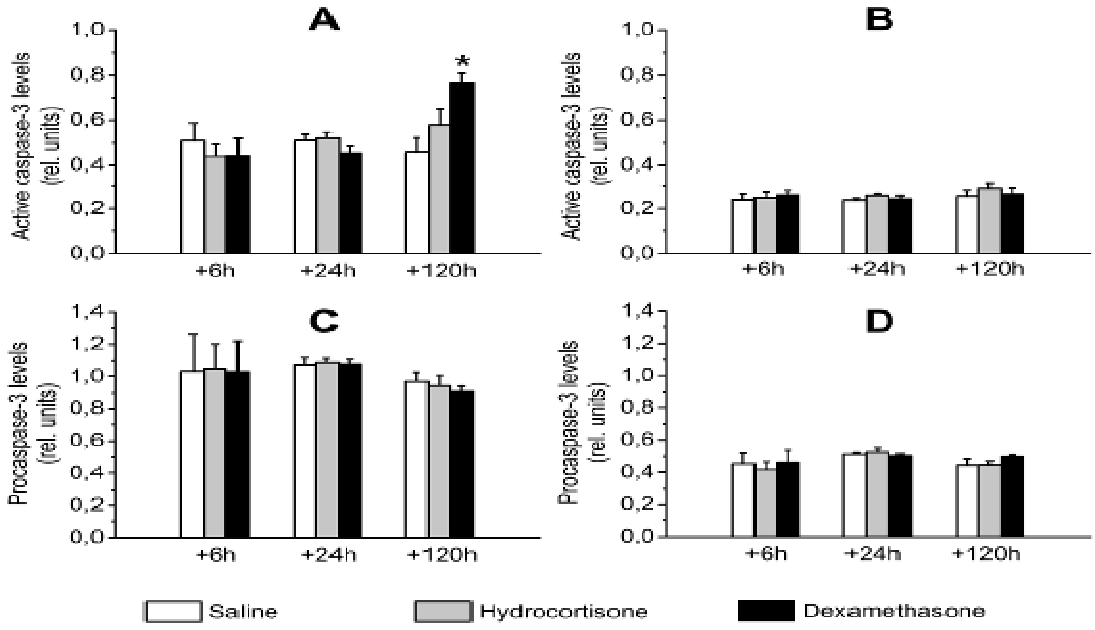

Figure 2. Expression of caspase-3 enzyme forms in the developing brain of neonatal rats in 6, 24 and 120 hours after injection of hydrocortisone and dexamethasone. Active caspase-3 levels in the cortex (A) and brainstem (B). Procaspase-3 levels in the cortex (C) and brainstem (D). * - p<0.001 vs saline-treated animals of the same age.

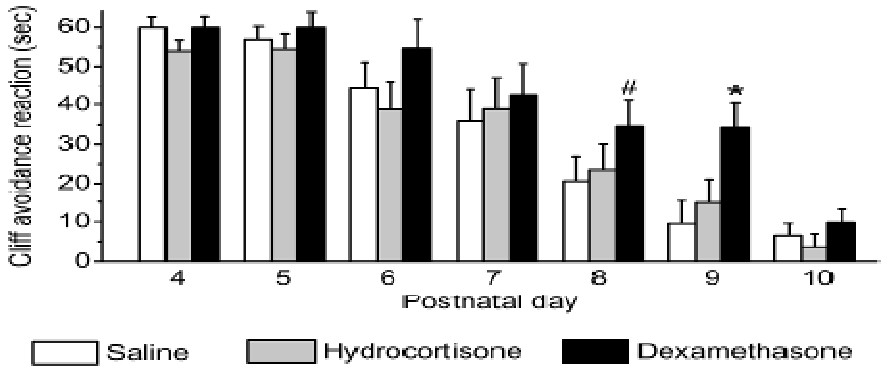

Figure 3. Development of cliff avoidance reaction * - p<0.05 vs saline-treated animals of the same age. # - p<0.1 vs saline-treated animals of the same age.

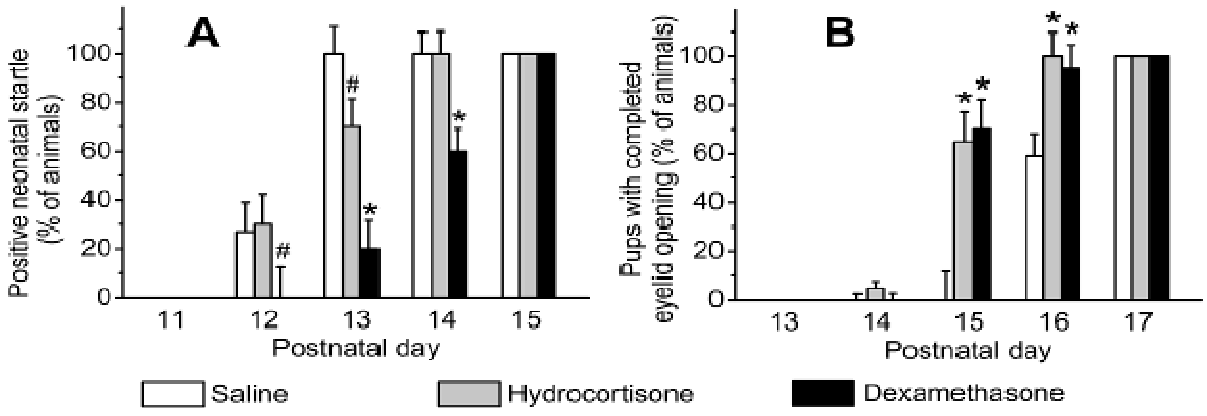

Figure 4. Developmental milestones after injection of hydrocortisone and dexamethasone. Development of neonatal startle reaction (A). Development of eyelid opening (B). * - p<0.05 vs saline-treated animals of the same age. # - p<0.1 vs saline-treated animals of the same age.

Conclusions

In summary, the results of the present study evidence that administration of low dose dexamethasone was still associated with the developmental abnormalities in behavior and changes in the expression of active caspase-3. However, the injection of equivalent dose of hydrocortisone was followed only by a delay in the body weight gain and a speed-up of the eyelid opening, and no behavioral and molecular changes were observed in rats received this drug. Thus, lowering the dose of dexamethasone is not a therapeutic option, and other corticosteroids, such as hydrocortisone, should be considered to be used to prevent the respiratory distress syndrome in neonates.

Acknowledgements

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