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Dexamethasone administration to the neonatal rat results in neurological dysfunction at the juvenile stage even at low doses

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ABSTRACT

Dexamethasone (DEX), a synthetic glucocorticoid, has been widely used to prevent the development of a variety of poor health conditions in premature infants including chronic lung disease, inflammation, circulatory failure, and shock. Although there are some reports of neurologic complications related to DEX exposure, its full effects on the premature brain have not been examined in detail. To investigate the effects of DEX on neural development, we first administered low doses (0.2 mg/kg bodyweight or less) of the glucocorticoid to neonatal rats on a daily basis during the first postnatal week and examined subsequent behavioral alterations at the juvenile stage. DEX-treated rats exhibited not only a significant reduction in both somatic and brain weights but also learning disabilities as revealed in the shuttle avoidance test. The hippocampi of DEX-treated rats displayed a high apoptotic and a low mitotic cell density compared to control rats on day 7 after birth. In a subsequent experiment, neural stem/progenitor cells were cultured in the presence of DEX for 6 days. The glucocorticoid inhibited cell growth without an increase in cell death. These results suggest that administration of DEX to premature infants induces neurological dysfunction via inhibition of the proliferation of neural stem/progenitor cells.

1. Introduction

Postnatal synthetic glucocorticoid therapy, especially DEX therapy, has been widely used to prevent chronic lung disease in premature newborns [1]. Newborn mortality, especially in relation to extremely low birth weight, has been expected to be reduced due to DEX therapy by decreasing of severe CLD. However, there is increasing evidence suggesting disadvantages of early DEX administration to premature neonates, including bodyweight loss, reduced height, and neurodevelopmental disorders [2,3], in addition to loss of brain volume [4]. Moreover, 8 randomized controlled studies enrolling 679 infants revealed a significant risk for neurodevelopmental abnormalities and cerebral palsy after early DEX treatment [5]. Similar disadvantages such as growth retardation and neurologic abnormalities have been induced in experimental animals, largely in the rat, by DEX administration during the neonatal period [6]. It is also of interest to understand whether early DEX administration affects learning abilities later in life, although this matter remains unresolved.

Thus, there are many reports concerning the disadvantages of early DEX treatment, and mechanisms underlying abnormal development of DEX-administered infants have been investigated using various experimental systems both *in vivo* and *in vitro*. Neonatal DEX exposure causes mild hyperactivity and cerebellar stunting [7], as well as selective apoptotic death of cerebellar neural progenitor cells in the rat [8]. Loss of pyramidal neurons or cells in the subgranular zone in the hippocampus is also observed following DEX administration in the neonatal rat [9]. Limited apoptosis and extensive sub-lethal damage were reported in the striatum as well [10]. In cell culture studies, DEX has been shown to exert undesirable effects on neurogenesis [6,11]. It is noteworthy that doses of DEX used in many of these *in vivo* and *in vitro* studies were relatively high (0.5 mg/kg bodyweight or more) compared with doses clinically used. To better understand the mechanisms governing developmental disorders induced by DEX administration, an experimental system should be established at DEX concentrations comparable to those used in neonatal DEX therapy for premature infants. Further, using this particular system, the effects of DEX on developmental parameters should be examined multilaterally both *in vivo* and *in vitro*. In

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the present work, we intended to know the influence of the treatment to neonatal rats with even at low dose of DEX in learning disabilities at a juvenile age and histological changes in the rat brain. Finally, the effects of low doses of DEX on the physiology of neural stem/progenitor cells were determined.

2. Materials and methods

2.1. DEX administration to rat pups

DEX (dexamethasone, water soluble) was purchased from Sigma (St. Louis, MO, USA). DEX was dissolved in phosphate-buffered saline (PBS) at a concentration of 9.8 μg/ml. Pregnant Sprague–Dawley (SD) rats at gestational day 7 were obtained from SLC (Shizuoka, Japan) and maintained under a 12-h light/dark cycle (lights on from 8:00 A.M. to 8:00 P.M.) with *ad libitum* access to food and water. All dams delivered pups on day 22 of gestation (assigned to postnatal day 0; P0). After birth, the bodyweight of each rat was measured every day during the first week, and the bodyweight and brain wet weight were measured on P7, P14, and P36. Pups were divided into two groups on P1: a control group and a DEX-treated group. DEX was injected intraperitoneally to pups at a concentration of 0.2 mg/kg bodyweight daily from P1 to P3, then 0.1 mg/kg daily from P4 to P7 during the light period [12]. Control pups received only the vehicle. All experiments were conducted with protocols approved by Nagoya University and Aichi Medical University Animal Experimental Committees. All efforts were made to minimize the number of animals used and their suffering.

2.2. Tissue preparation for histology

Rats were anesthetized with ether and brains were fixed via perfusion with 0.1 M phosphate buffer, pH 7.2, containing 4% paraformaldehyde on P7 or P14. Brains were removed and post-fixed with 95% alcohol containing 5% acetic acid, then embedded in paraffin. Six-micrometer-thick coronal sections were obtained. Sections were deparaffinized by a sequential treatment with xylem, followed by 100%, 95%, and 70% alcohol, each for 5 min, then rinsed with PBS for 10 min. Some of the sections were stained with hematoxylin and eosin.

2.3. Immunohistochemistry

Deparaffinized and hydrated sections were heated in boiling 0.01 M citrate buffer, pH 6.0, for 10 min, then allowed to cool. Blocking was performed using 4% normal donkey serum in PBS for 60 min. The sections were incubated with a primary antibody at 4 °C overnight. We used two primary antibodies, an antibody against cleaved caspase-3 (rabbit IgG; 1:50 dilution; Cell Signaling, Danvers, MA) to detect apoptotic cells, and an antibody against phosphorylated histone H3 (Ser10) (rabbit IgG; 1:300; Merck, Billerica, MA) to detect actively mitotic cells. On the following day, sections were incubated with a biotinylated donkey anti-rabbit IgG antibody (1:400; Jackson ImmunoResearch, West Grove, PA) for 60 min at room temperature. To block endogenous peroxidase activity, sections were incubated with 3% H_2O_2 for 5 min and washed with PBS for 10 min. Subsequently, sections were incubated with Elite ABC reagent (Vectastain, Burlingame, CA) for 60 min. The immunocomplex formed was visualized by immersion in diaminobenzidine (Merck, Billerica, MA)/PBS. After being mounted with Entellan (Merck), sections were examined using an inverted microscope (IX71; Olympus, Tokyo, Japan).

2.4. Evaluation of immunopositive cell densities

Immunopositive cells in the left hippocampal area (see Fig. 5a) were counted under a microscope using the image analysis software WinROOF (Mitani, Tokyo, Japan). The area of the hippocampus was also determined using the same image analysis software. Density of immunopositive cells was expressed as cell numbers per mm^2 of the hippocampus.

2.5. Behavioral analyses

Two kinds of behavioral tests, an open-field test and a shuttle avoidance test, were performed during the juvenile period (from 4 to 6 weeks) by the same experimenter, starting at 10:00 P.M. Both tests were carried out according to methods and using the same equipment as described previously [13] with some minor modifications as follows.

2.5.1. Open-field test

Each rat was placed into a white 90-cm cube with every 30-cm grid on the base, and behavior was observed and recorded by a video camera for 180 s. The test was performed once a day for four consecutive days. The following parameters were assessed by this test: total movement, center entries, and rearing.

2.5.2. Shuttle avoidance test

Each rat underwent 20 sessions of a shuttle avoidance test each day for four consecutive days. The test was conducted in an automated shuttle box subdivided into two compartments with independently electrified stainless-steel bars as floors (Med Associates Inc., St. Albans, VT). The data were analyzed with the MED-PC IV program. By this test, the following four parameters can be assessed: total activity, mean escape latency, mean avoidance latency, and avoidance rate. The avoidance rate was expressed as a percentage of successful avoidance trials in 20 sessions, such that it could be considered a parameter representing learning ability.

2.6. Neurosphere culture of neural stem/progenitor cells

Neural stem/progenitor cells were cultured and expanded as neurospheres by a method described previously [14]. In brief, telencephalons were excised from E14 rat fetuses and mechanically dissociated by pipetting into a single cell suspension in the presence of 0.01% DNase I (Roche Applied Science, Tokyo, Japan). Cells were plated in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (1:1) mixture medium (Invitrogen, Carlsbad, CA) containing N2 and B27

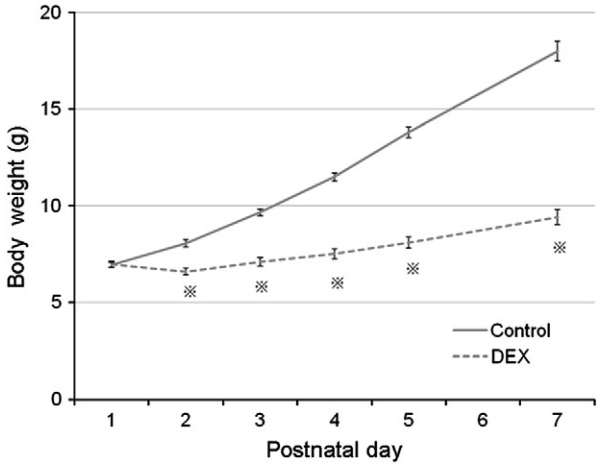

Fig. 1. Effect of dexamethasone administration on growth of neonatal rats. Dexamethasone (DEX) was administered intraperitoneally to neonatal rats at a dose of 0.2 mg/kg bodyweight on postnatal days 1, 2, and 3, and 0.1 mg/kg bodyweight on days 4, 5, 6, and 7. Their bodyweights were measured every day from birth to postnatal day 7. Control rats were treated only with the vehicle. Error bars represent the S.E.M. $n=6$ for control, 12 for DEX-treated rats. $^*p<0.001$.

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