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BRAIN RESEARCH

Research Report

Glucocorticoids dexamethasone and hydrocortisone inhibit proliferation and accelerate maturation of chicken cerebellar granule neurons☆

Petra Aden[a,*], *Ragnhild E. Paulsen*[b], *Jan Mæhlen*[a], *Else Marit Løberg*[a], *Ingeborg L. Goverud*[a], *Knut Liestøl*[c], *Jon Lømo*[a]

[a]*Department of Pathology, Oslo University Hospital, Ullevål, Norway*
[b]*Department of Pharmaceutical Biosciences, School of Pharmacy, University of Oslo, Norway*
[c]*Department of Informatics, University of Oslo, Norway*

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ABSTRACT

Glucocorticoid (GC) treatment in premature infants may have detrimental effects on the immature brain. Here we show that GCs dexamethasone (Dex) and hydrocortisone (HC) reduce proliferation and induce differentiation of chicken embryo cerebellar neurons in vivo and in vitro. Granule neurons incorporating bromodeoxyuridine were reduced in the internal granular layer (IGL) after 24-h exposure to both substances on embryonic day 17, with Dex about 100-fold more potent than HC. The effects were blocked by GR antagonist RU 38486. Both GCs also increased the expression of neuronal differentiation markers microtubule-associated protein 2 (Map2) and neuronal nuclei protein (NeuN), measured by western blotting of whole cerebellar lysates and immunohistochemistry, respectively. Treatment of cerebellar granule neuron cultures with both GCs significantly reduced the percentage of proliferating-cell nuclear antigen (PCNA) positive neurons and increased NeuN positive neurons, with similar dose–response relationship as in vivo. The cytostatic agent cytosine arabinoside showed comparable effects both on proliferation and differentiation. In conclusion, the effects of Dex and HC on chicken cerebellar granule neuron proliferation are GR mediated and reflect their pharmacological potency. In addition, the effects on differentiation may be related to a cell cycle block per se, since cytosine arabinoside mimicked the effect of the GCs.

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* *Corresponding author at:* Section of Neurology, Department of Pediatrics, Oslo University Hospital, Ullevål, PO Box 4956 Nydalen, N-0424 Oslo, Norway. Fax: +47 22226628.
E-mail address: petra.aden@oslo-universitetssykehus.no (P. Aden).

Abbreviations: AraC, Cytosine arabinoside; BrdU, Bromodeoxyuridine; CCGN, Chicken cerebellar granule cell; DAB, Diaminobenzidine; Dex, Dexamethasone; DIV, Days in vitro; EGL, External granular layer; GC, Glucocorticoid; HC, Hydrocortisone/cortisol; IGL, Internal granular layer; Map2, Microtubule-associated protein 2; NeuN, Neuronal nuclei protein; PCNA, Proliferating cell nuclear antigen

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1. Introduction

Antenatal treatment with glucocorticosteroids (GCs) accelerates lung maturation in premature infants. GCs are also used in treatment of bronchopulmonary dysplasia and a variety of other complications that affect premature infants after birth. In recent years evidence has accumulated that postnatal GC treatment of premature infants may cause cerebral palsy and more subtle motor deficiencies (Doyle et al., 2005; O'Shea et al., 1999; Yeh et al., 2004). One of the target organs of GCs may be the developing cerebellum, as indicated by studies in several animal models (Aden et al., 2008; Heine and Rowitch, 2009; Noguchi et al., 2008).

Brain development is an intricately complex process involving cell proliferation, programmed cell death (apoptosis) and differentiation. We have previously used a chicken embryo model to show that a single dose of dexamethasone (Dex) and hydrocortisone (HC) injected on embryonic day (E)16 increases apoptosis in cerebellar granule neurons, both in vivo and in vitro (Aden et al., 2008). The effect of Dex and HC was rapid and the agents showed equal potency, indicating a non-classical (non-transcription dependent) mechanism of action. Dex-induced neuronal apoptosis in the external granular layer (EGL) of the cerebellum has also been reported in mice (Noguchi et al., 2008). Additionally, GCs are known to promote cell death in neurons in other parts of the brain (Howard et al., 1969; Uno et al., 1990; Yu et al., 2010). Postnatal cortisone treatment reduced DNA content in adult mice cortex (Howard et al., 1969). Dex led to a reduction of hippocampal pyramidal cells and granule neurons of the dentate gyrus in newborn rhesus monkey (Uno et al., 1990) when given prenatally and rat (Yu et al., 2010) when given postnatally.

Cell proliferation and apoptosis are opposing determinants of brain growth, and the former is the focus of the present study. Using BrdU labeling we have studied the effect of Dex and HC treatment of chicken embryos on cerebellar granule neuron proliferation in the IGL, and further extended the investigation to purified cerebellar granule neurons in vitro. A significant inhibition of proliferation was demonstrated. Interestingly, this effect was coupled to neuronal differentiation, as measured by an increased expression of neuronal maturation markers, Map2 and NeuN. The antimetabolite and cell cycle inhibitor cytosine arabinoside (AraC) gave a similar differentiation response.

2. Results

2.1. *Dex and HC reduce the number of proliferating neurons in cerebellum*

We investigated cell proliferation in the cerebellar IGL using BrdU labeling. Chickens on E16 were treated with different dosages of Dex and HC (0.01 mg, 0.1 mg, 1 mg or 5 mg/kg egg) for 24 h, and BrdU was added 3 h before sacrifice. CCGN is the dominating cell type in the IGL. Only a few cells stain for the glia markers GFAP and S-100 (unpublished results). Moreover, doublestaining with the neuron marker Map2 showed that most BrdU incorporating cells also expressed Map2 (Fig. 1A). Both Dex and HC reduced the percentage of proliferating cells relative to all cells in the IGL in a dose-dependent manner (Figs. 1B and C). Statistically significant reduction in proliferation occurred at 0.1 mg/kg for Dex and at 5 mg/kg for HC, consistent with a ~50-fold difference in potency. The maximum level of reduction of proliferation was the same for HC and Dex, approximately 40%. To investigate if the effect of Dex and HC was mediated by GR, eggs were pretreated with GR antagonist RU 38486 (20 mg/kg egg) for 3 h before treatment with Dex and HC (5 mg/kg egg). As shown in Fig. 2, inhibition of cell proliferation was blocked.

2.2. *Dex and HC increase the number of CCGNs expressing differentiation markers NeuN and Map2 in the embryo cerebellum*

Reduced proliferation may be coupled with differentiation in immature cells, and for this reason we examined the expression of two important neuronal markers NeuN and Map2. Map2 protein is generally accepted as a marker for dendritic outgrowth and consequently of mature neurons (Buard et al., 2010; Lind et al., 2005), whereas NeuN is a postmitotic neuronal marker that localizes to the nuclear matrix (Dent et al., 2010; Kim et al., 2009). NeuN expression was studied by immunohistochemical staining of cerebellar slides after Dex and HC treatment. Both Dex and HC increased the fraction of NeuN positive cells in the IGL from 50% to 70% (Fig. 3A). Representative photomicrographs of Map 2 immunohistochemical staining are shown (Fig. 3B, left panel). Map2 could not be accurately quantified immunohistochemically in sections due to expression primarily in neurites, not cell bodies. We therefore used western blotting of total homogenated cerebellar lysates. Treatment with Dex significantly increased relative expression of Map2 protein (Fig. 3B, right panel). HC treatment increased mean Map2 expression, but the result was not statistically significant.

2.3. *Effect of Dex and HC on proliferation and differentiation is mimicked by AraC in cell culture*

CCGN cultures were treated with Dex and HC. A low dose of Dex (0.1 μM) was chosen because Dex in a high dose (10 μM) induces cell death of 50% of cells in culture 6. Dex in a dose of 0.1 μM induced much lower cell death rates (8.5%). Treatment with Dex (0.1 μM) significantly reduced the percentage of proliferating cells, as measured by positivity for the proliferation marker PCNA (Figs. 4A and B). The reduction detected with HC (10 μM) was less and not statistically significant. The effect of Dex was almost as potent as a treatment with the cytostatic agent AraC, used at 10 μM. This concentration of AraC did induce cell death of about 20% (unpublished results).

Differentiation was examined in vitro by measuring expression of NeuN. Dex (0.1 μM) significantly increased the percentage of NeuN positive neurons (Figs. 5A and B). HC (10 μM) also increased the mean percentage of positive cells, but the result was not statistically significant. AraC treatment showed the same magnitude of effect as Dex. Both AraC and GCs prolong the G1 phase of the cell cycle, but have otherwise no known common mode of action. The analogous effects of GCs and AraC indicate that increased differentiation most likely is the effect of disturbances of the neuronal cell cycle and not an effect on the postmitotic neuron.