Glucocorticoid effects on neuronal proliferation, cell death and differentiation in the immature nervous system

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“Learning without thinking is of no use. 
Thinking without learning is dangerous.”

(Confucius)
Acknowledgements

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<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>11β-HSD</td>
<td>11β-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptotic peptidase activating factor 1</td>
</tr>
<tr>
<td>AraC</td>
<td>Cytosine arabinoside</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2 associated X protein</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BPD</td>
<td>Bronchopulmonal dysplasia</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>CBG</td>
<td>Corticosteroid binding globulin</td>
</tr>
<tr>
<td>CCGN</td>
<td>Chicken cerebellar granule neuron</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin releasing hormone</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
</tr>
<tr>
<td>Dex</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>DIV</td>
<td>Day in vitro</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>EGL</td>
<td>External granular layer</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ELBW</td>
<td>Extreme low birth weight</td>
</tr>
<tr>
<td>Fgf8</td>
<td>Fibroblast growth factor 8</td>
</tr>
<tr>
<td>Gbx2</td>
<td>Gastrulation brain homeobox 2</td>
</tr>
<tr>
<td>GC</td>
<td>Glucocorticoid</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GRE</td>
<td>Glucocorticoid responsive element</td>
</tr>
<tr>
<td>HC</td>
<td>Hydrocortisone/Cortisol</td>
</tr>
<tr>
<td>IGL</td>
<td>Internal granular layer</td>
</tr>
<tr>
<td>IQ</td>
<td>Intelligence quotient</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal axis</td>
</tr>
<tr>
<td>LBW</td>
<td>Low birth weight</td>
</tr>
<tr>
<td>luc</td>
<td>Luciferase</td>
</tr>
<tr>
<td>Map2</td>
<td>Microtubule-associated protein 2</td>
</tr>
<tr>
<td>Math1</td>
<td>Meprin associated Traf homology domain 1</td>
</tr>
<tr>
<td>mGR</td>
<td>membrane bound GR</td>
</tr>
<tr>
<td>ML</td>
<td>Molecular layer</td>
</tr>
<tr>
<td>MMTV-HRE</td>
<td>Mouse mammary tumor virus hormone responsive element</td>
</tr>
<tr>
<td>MR</td>
<td>Mineral corticoid receptor</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NBRE</td>
<td>NGFI-B response element</td>
</tr>
<tr>
<td>NeuN</td>
<td>Neuronal nuclei</td>
</tr>
<tr>
<td>NGFI-B</td>
<td>Nerve growth factor induced clone B</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>NR</td>
<td>Nuclear Receptor</td>
</tr>
<tr>
<td>Nurr1</td>
<td>Nuclear receptor related protein 1</td>
</tr>
<tr>
<td>NOR1</td>
<td>Neuron derived orphan receptor 1</td>
</tr>
<tr>
<td>NurRE</td>
<td>Nur response element</td>
</tr>
<tr>
<td>Otx2</td>
<td>Orthodenticle homeobox 2</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>POMC</td>
<td>Proopiomelanocortin</td>
</tr>
<tr>
<td>PL</td>
<td>Purkinje cell layer</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>Ptf1A</td>
<td>Pancreas specific transcription factor 1A</td>
</tr>
<tr>
<td>RDS</td>
<td>Respiratory distress syndrome</td>
</tr>
<tr>
<td>RI</td>
<td>Renilla</td>
</tr>
<tr>
<td>Rsv</td>
<td>Rous sarcoma virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>tk</td>
<td>Thymidine kinase</td>
</tr>
<tr>
<td>VLBW</td>
<td>Very low birth weight</td>
</tr>
<tr>
<td>Wnt1</td>
<td>Wingless-related MMTV integration site 1</td>
</tr>
</tbody>
</table>
Original papers

This doctoral thesis is based on the following publications:

Paper 1
*Chicken cerebellar granule neurons rapidly develop excitotoxicity in culture.*
J Neurosci Methods, 2006 30; (156): 129-135

Paper 2
*Low-potency glucocorticoid hydrocortisone has similar neurotoxic effects as high-potency glucocorticoid dexamethasone on neurons in the immature chicken cerebellum.*
Brain Res, 2008 1236:39-48

Paper 3
Strøm BO*, Aden P*, Mathisen GH*, Lømo J, Davanger S, Paulsen RE
*Transfection of chicken cerebellar granule neurons used to study glucocorticoid receptor regulation by nuclear receptor 4A (NR4A)*

Paper 4
*Glucocorticoids dexamethasone and hydrocortisone inhibit proliferation and accelerate maturation of chicken cerebellar granule neurons.*
Manuscript

*Authors contributed equally to this study
1. Introduction

1.1 Prematurity and its complications

Prematurity is defined as birth before 37 weeks of gestation and may be grouped according to either weight or gestational age (Tab. 1, 1).

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Weight Range</th>
<th>Gestational Weeks</th>
<th>Frequency</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low birth weight (LBW)/ Moderately preterm</td>
<td>1500 - 2500 grams</td>
<td>32 - 37 gestational weeks</td>
<td>2526</td>
<td>4.1%</td>
</tr>
<tr>
<td>Very low birth weight (VLBW)/ Very preterm</td>
<td>1000 - 1499 grams</td>
<td>28 - 32 gestational weeks</td>
<td>343</td>
<td>0.6%</td>
</tr>
<tr>
<td>Extremely low birth weight (ELBW)/ Extremely preterm</td>
<td>500 - 999 grams</td>
<td>22 - 27 gestational weeks</td>
<td>235</td>
<td>0.4%</td>
</tr>
<tr>
<td><strong>Total number of births in Norway in 2009</strong></td>
<td></td>
<td></td>
<td>62213</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table 1. Subgroups in the premature population and their frequency in the total number of births in Norway in 2009 (2)

Over the last five decades a picture of decreasing mortality has emerged for all groups of premature infants (Fig. 1, 3). The application of early intubation and more sophisticated fetal monitoring, the introduction of surfactant treatment and the use of incubators stabilizing the environment, the introduction of prenatal GC treatment of mothers in the 1990s are some of the factors behind this positive trend (4, 5).

The mortality of the extreme preterm group can also vary between hospitals and countries. For babies with birth weight 600-699 grams mortality ranges from 27 to 63 % in centers in industrialized countries (5, 6).

Advances in minimal invasive treatment and nursing (Newborn individualized Developmental Care and Assessment Program - NIDCAP, 7) have reduced environmental stress factors like pain sensations because of frequent blood tests and unpleasant auditory sensations because of frequent alarms on the neonatal intensive care unit.
1.1.1 Morbidity associated with premature birth

Morbidity of the preterm child in the postnatal period (28 days after birth) involves most organ systems (Tab. 2).

Treating the extreme low birth weight infant is challenging. The patient may need intubation, artificial ventilation and total parental nutrition in the initial period of treatment, and intensive care is needed over several weeks to months. Nevertheless, even with recent advances in treatment the prevalence of bronchopulmonary dysplasia (BPD), necrotizing enterocolitis and severe intraventricular hemorrhage remains relatively stable in recent cohorts (1995-2000) of preterm infants with BW < 1500 grams (9).
<table>
<thead>
<tr>
<th>Morbidity</th>
<th>Frequency for 501-1500 grams of birth weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth failure</td>
<td>91</td>
</tr>
<tr>
<td>Respiratory distress syndrome</td>
<td>44</td>
</tr>
<tr>
<td>Patent ductus arteriosus</td>
<td>29</td>
</tr>
<tr>
<td>Intraventricular hemorrhage</td>
<td>27</td>
</tr>
<tr>
<td>Late onset septicemia</td>
<td>22</td>
</tr>
<tr>
<td>Bronchopulmonary dysplasia</td>
<td>22</td>
</tr>
<tr>
<td>Necrotizing enterocolitis</td>
<td>7</td>
</tr>
<tr>
<td>Pneumothorax</td>
<td>5</td>
</tr>
<tr>
<td>Periventricular leukomalacia</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 2. Preterm morbidity for birth weight 501-1500 grams adapted from Fanaroff et al. 2007 (9).

*Growth failure:* <10th percentile at 36 weeks postconceptual age

### 1.1.2 Long term consequences of prematurity

Several studies have found that prematurity is associated with significantly lower height and weight as well as lower head circumference in early childhood and even into young adulthood associated with prematurity (10, 11, 12). The main focus in long term follow up after premature birth has been on impaired neurodevelopment and its consequences for cognitive, motor, neurosensory, behavioral and functional outcome. According to a Swedish cohort study from 1959 to 2002 (13, 14) the prevalence of cerebral palsy, a non-progressive disorder of movement and posture as a result of an injury to the immature brain, has slightly increased (Fig. 2) for all groups. The most recent cohort (1999-2002) showed, though, a significant decrease in CP for the ELBW group, compared to the previous cohort (1995-1998). Other studies have reported similar findings (15). The recent increase in CP for the term born cohort mainly accounted for the general increase in CP in the Swedish study group. It was speculated that this increase was caused by an increasing survival of term babies suffering from hypoxic-ischemic encephalopathy, an injury of the brain caused by hypoxia and ischemia ante- and intrapartum (14).
Figure 2. Prevalence of cerebral palsy in a Swedish population according to Himmelmann et al. (14), CP cerebral palsy

The incidence for other disabilities such as mental retardation, epilepsy, blindness and moderate to severe hearing impairment has been rather stable over the past 20 years (5). A cohort study of all live born individuals from 1967-1983 in Norway concluded that gestational age at birth was inversely correlated to the incidence of cerebral palsy, mental retardation, autism spectrum disorders as well as other psychological or psychiatric disorders (16). The total disability rate was highest in the ELBW group. Preterm birth was also associated with lower levels of education, lower job income, and reduced probability to have children and be married, even in the absence of a major medical disability. This association was also found in other studies of social outcomes of prematurity (17).

Recent studies of preterm infants of birth cohorts born in the 1990’s show reduced school performance and lower IQ (18, 19, 20, 17) indicating that subnormal neurodevelopment is persisting even in the newer cohorts. Recent cohort studies did not include postnatal GC treatment in their analysis of confounding factors. There is also evidence that prematurity might be associated with other major disease complexes in adulthood, such as hypertension and diabetes type II (21, 22).
1.1.3 GC regulation and the preterm infant

GCs are secreted from the adrenal gland and are called “stress hormones”. GCs were found to have a vital role in maintaining processes like salt regulation, blood pressure and temperature regulation. Adrenalectomized patients died without substitution of GCs (23). Later, the connection between stress and amelioration in autoimmune disease or allergy was recognized (24). GC treatment in autoimmune disease led to the discovery of a range of side effects on different organs such as changes in glucose and insulin level, bone, fluid and electrolyte balance and depression.

The existence of a distinct feedback mechanism for regulation of the glucocorticoids (GCs) in connection with stress, the hypothalamic-pituitary-adrenal axis (HPA axis) was first described of H. Selye (25). The paraventricular nucleus of the hypothalamus produces corticotropin releasing factor (CRH), which in turn promotes release of adrenocorticotropic hormone (ACTH) from the anterior part of the pituitary. ACTH reaches the adrenal gland and releases GCs from the outer zone. GCs in turn block the release of ACTH through a negative feedback mechanism on proopiomelanocortin (POMC), the precursor of ACTH. Studies in rat show evidence for a non-functioning GC homeostasis in fetal life. High levels of circulating steroids fail to downregulate glucocorticoid receptor (GR) in the fetal brain, thereby leaving it more vulnerable to excess GC levels occurring in stressful situations (26). Studies of animals and human fetuses in the last term of gestation showed that GC production increases near parturition (27) and is necessary for parturition, as ablation of the fetal paraventricular nucleus (production of CRH) leads to prolonged gestation (28, 29).

The fetal HPA axis is repressed by maternal hydrocortisone (HC, also called cortisol and the predominant GC in humans) circulating in the fetus. In late pregnancy increasing 11β-hydroxysteroid dehydrogenase 2 levels in the placenta lead to inactivation of maternal HC and liberate the fetal axis to produce GCs. In extreme preterm birth the infant is parted from the provider of HC and the immature adrenal gland is not able to produce enough HC. Thus a relative adrenal insufficiency is present in premature infants (30).
1.2 Glucocorticoid (GC) treatment in premature infants and long-term neurodevelopmental consequences

Experimental pharmacological treatment of premature infants for the prevention of neonatal respiratory distress syndrome (RDS), characterized by respiratory distress, increasing oxygen requirement and diffuse reticular-granular infiltrates as well as atelectasis on chest x-ray, started in the 1950s. Studies from this era showed no clear treatment effect of GCs on RDS, but most studies included only small numbers of treated individuals (31, 32). In a large study 135 infants were included but there was no control group established (33).

Based on experiments on prenatal lung development in lamb Liggins and Howie (34) suggested that prenatal treatment with highly potent GCs might accelerate lung maturation. Their later clinical "trial of ante partum glucocorticoid treatment for prevention of RDS in premature infants" (35) was the first that could show significantly reduced incidence of RDS in preterm babies of mothers treated with 12 mg betamethasone when compared to preterm babies of mothers treated with 6mg cortisone (Cort). Cort has a 70 times lower GC potency than betamethasone and served as control substance. Subsequently several controlled studies confirmed these results (Roberts D 2010, Fig.3). A first systematic review in 1990 concluded that antenatal GC treatment resulted in substantially reduced neonatal mortality and morbidity (36). Finally this lead to general recommendations and international guidelines for steroid use in treatment of preterm infants (37, 38).
Figure 3. Meta-analysis of risk ratio for RDS with antenatal GC treatment according to Roberts et al. 2010 (39). RDS risk ratio for individuals treated with antenatal GCs compared to controls is listed for 21 studies. Total risk ratio and test for overall treatment effects are highlighted (red).

Risk assessment for cerebral palsy in childhood showed a decreased risk with antenatal GC treatment (39).

With the survival of more preterm infants a new disease entity emerged: bronchopulmonary dysplasia (BPD) characterized by pulmonal interstitial fibrosis, pulmonary opacities on x-ray and oxygen dependency at 36 gestational weeks. Eventually BPD was linked to a combination of factors: prematurity, mechanical ventilation, high oxygen exposure and inflammation. The inflammation hypothesis prompted studies that used GCs for the postnatal prevention of BPD (for review see 40,
Reduced risk for BPD was found with early and late Dex treatment, but not HC treatment.

### Figure 4A. Meta-analysis of risk ratio for BPD at 36 weeks in survivors with early (<8 days) GC treatment according to Halliday et al. 2009 (40). BPD risk ratio for individuals treated with postnatal Dex or HC compared to controls is listed for 18 studies. Total risk ratio and test for overall treatment effects are highlighted (red).
Figure 4B. Meta-analysis of risk ratio for BPD at 36 weeks in survivors with late (>7 days) GC treatment (Dex only) treatment according to Halliday et al. 2009 (41) BPD risk ratio for individuals treated with postnatal Dex compared to controls is listed for 4 studies. Total risk ratio and test for overall treatment effects are highlighted (red).

The Cochrane Collaborative Study Group also summarized the relative risk for cerebral palsy estimated by randomized controlled clinical studies of early (<8 days) postnatal GCs (40) and late (>7 days) postnatal GC treatment for the prevention of BPD (41). Early treatment (< 8 postnatal days) of premature infants was found to give an increased risk of cerebral palsy (Fig 5A, 40). Dexamethasone (Dex) treatment accounted for the increased risk ratio (1.82) alone whereas HC treatment showed no increased risk ratio for CP in survivors (0.95). Late treatment (>7 days of age) with GCs (only Dex used) did not change the cerebral palsy risk rate significantly (Fig. 5B, 41) thereby indicating that GC damage to the brain may be more significant at an early developmental stage. A randomly controlled trial by Yeh et al. (42) on preterm infants treated with Dex for BPD in 28 days postnatal showed significantly reduced IQ and motor skills at school age compared to preterm controls, who were not treated with Dex. Guidelines from the American Academy of Pediatrics, the Canadian Paediatric Society and the European Association of Perinatal Medicine have since concluded that postnatal GC should be avoided in the first 4 postnatal days and should be reserved for the treatment of the sickest preterm infants dependent on ventilator (43).
### Figure 5A: Meta-analysis of risk ratio for CP in survivors with early (<8 days) GC treatment according to Halliday et al. 2009 (40). CP risk ratio for individuals treated with postnatal Dex or HC compared to controls is listed for 18 studies. Total risk ratio and test for overall treatment effect are highlighted (red).

<table>
<thead>
<tr>
<th>Study/Author</th>
<th>Steroid n/N</th>
<th>Control n/N</th>
<th>Risk Ratio</th>
<th>Weight</th>
<th>Risk Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Desmopressine</td>
<td>222</td>
<td>322</td>
<td>4.7%</td>
<td>0.64 [0.12, 2.46]</td>
<td></td>
</tr>
<tr>
<td>Sands et al. 1994</td>
<td>39</td>
<td>17</td>
<td>1.7%</td>
<td>0.27 [0.08, 2.12]</td>
<td></td>
</tr>
<tr>
<td>Stirnweil 1996</td>
<td>10792</td>
<td>10490</td>
<td>18.3%</td>
<td>0.11 [0.01, 1.81]</td>
<td></td>
</tr>
<tr>
<td>Sekiro 2000</td>
<td>47</td>
<td>120</td>
<td>1.6%</td>
<td>0.31 [0.04, 2.33]</td>
<td></td>
</tr>
<tr>
<td>Stark 2001a</td>
<td>1176</td>
<td>1167</td>
<td>19.6%</td>
<td>0.21 [0.03, 1.27]</td>
<td></td>
</tr>
<tr>
<td>Sublec 2000</td>
<td>910</td>
<td>910</td>
<td>37%</td>
<td>0.02 [0.00, 0.47]</td>
<td></td>
</tr>
<tr>
<td>Walsh 2001</td>
<td>1277</td>
<td>1074</td>
<td>13.6%</td>
<td>0.14 [0.03, 0.58]</td>
<td></td>
</tr>
<tr>
<td><strong>Subtotal (95% CI)</strong></td>
<td>298</td>
<td>288</td>
<td>63.1%</td>
<td>1.82 [1.29, 2.57]</td>
<td></td>
</tr>
</tbody>
</table>

Total events: 75 (Steroid), 40 (Control)
Heterogeneity: Ch^2 = 12.23, df = 6 (P = 0.05); I^2 = 52%

Test for overall effect: Z = 3.41 (P = 0.00068)

| 2. Hydrocortisone | 213 | 212 | 1.6% | 1.65 [0.12, 17.84] |
| Bonar et al. 2007 | 219 | 214 | 35% | 0.074 [0.01, 4.61] |
| Bockhorn 2005 | 203 | 022 | 0.8% | 0.24 [0.04, 1.05] |
| Winterberg 1999 | 170 | 29 | 34% | 0.40 [0.10, 1.66] |
| Winterberg 2004 | 1612 | 1612 | 27.6% | 0.09 [0.04, 1.66] |

Subtotal (95% CI): 191 | 182 | 36.9% | 0.95 [0.56, 1.63] |

Total events: 23 (Steroid), 23 (Control)
Heterogeneity: Ch^2 = 2.17, df = 4 (P = 0.70); I^2 = 0%

Test for overall effect: Z = 0.13 (P = 0.89)

**Total (95% CI):** 489 | 470 | 100% | 1.50 [1.13, 2.00] |

Total events: 90 (Steroid), 63 (Control)
Heterogeneity: Ch^2 = 18.77, df = 11 (P = 0.07); I^2 = 41%

Test for overall effect: Z = 2.78 (P = 0.0055)
Figure 5B: Meta-analysis of risk ratio for CP in survivors with late (>7 days) GC treatment assessed at age 1-3 according to Halliday et al. 2009 (41). CP risk ratio for individuals treated with postnatal Dex compared to controls is listed for 18 studies. Total risk ratio and test for overall treatment effect are highlighted (red).

1.3 The need for animal models in the study of GC effects in immature organisms

Several animal models have been developed to study the effects and side effects of antenatal GC administration. Animal models are needed in the study of these effects because the evaluation of basic toxicological mechanisms such as apoptosis, proliferation and differentiation in the brain as a result of GC treatment cannot be accomplished in humans. Moreover, as the premature infant undergoes intensive treatment and might be affected by various morbidities (see section 1.1.1), it is obviously important to distinguish GC effects from other consequences of prematurity which may only be achieved in animal models to a sufficient degree (44, 45).

A large body of initial experimental work was done in sheep. Liggins et al. (34) were the first to test Dex as initiator of premature labor and observed partial aeration in the alveolar tissue of some newborn lambs of mothers treated with GCs. In subsequent studies single and repeated doses of prenatal administered GC increased lung compliance and surfactant protein B mRNA (46, 47). Studies in rhesus monkeys confirmed results from sheep studies (48).
A number of studies found reduced birth weight (46, 49, 50). Several studies show reduced brain weight with GC treatment. Table 3 summarizes results of representative experimental studies on how GC treatment affects brain size and structures. Permanent degeneration of pyramidal neurons in the hippocampus and to a lesser degree in other cortical regions in rhesus monkeys treated with a single dose or repeated doses of Dex in utero have been reported by Uno et al. (51). Increased apoptosis of neural progenitor cells and immature neurons in the hippocampus was shown in rat pups postnatal treated with Dex (52).

Cotterell et al. (53) found that cell proliferation in rat brain and especially in rat cerebellum was blocked by HC (approx. 40mg/kg) in rats. GC treatment of neonatal rats showed a permanent reduction of cell number in the adult cerebellum (54). Postnatal Dex treatment of mice resulted in a transient increased apoptosis of neural progenitor cells in the external granular layer (EGL) of the cerebellum (55).

Howard et al. (56) showed that Cort treatment caused reduced cerebellar size and “a lasting impairment in fine adjustment mechanisms of motor control” in mice. Ahlbohm et al. (57) showed that cerebellar granule neuron cultures from one-week old rat pups treated with Dex in pregnancy showed an impaired mitochondrial function and higher sensitivity to oxidative stress.

However, detailed molecular mechanisms for GC effects on cell death, proliferation and differentiation in the immature nervous system are still largely unknown.
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Table 3. Representative animal model studies of GC effects on the immature brain
1.4 The developing cerebellum as model structure for the study of GC effects

GC treatment of mice showed strongest effect on cell numbers in the cerebellum in one early study (58). During normal development DNA content (representing cell content) increased about 25% in postnatal mouse cerebrum, whereas the cerebellum multiplied its cell amount by approximately a factor of six (58). Cort treatment reduced the DNA amount in the cerebellum by 25% compared to 10% in cerebrum. Thus the cerebellum was the structure with the greatest structural changes on GC treatment. Cell birth, cell death and differentiation can be easily monitored. Both animal studies and clinical trials report effects on motor regulation (56, 42), thereby pointing to the cerebellum as a relevant model structure for the study of GC effects.

The avian cerebellum is organized in 10 folia (66). Early in development the cerebellum starts to form from the anterior-most rhombomere (a transiently divided segment in the hindbrain region of the embryo, that later becomes the rhombencephalon), rhombomere 1, as part of the metencephalon that engulfs the bottom of the fourth ventricle. The cerebellar structures expand dorsally and fuse to the dorsal area then outlining the roof of the fourth ventricle (Fig. 6, 67).
Figure 6. Early development of the cerebellum at about 4 weeks of development (a), at the end of the embryonic period (b) and at 13 weeks of development (c) according to ten Donkelaar et al. 2003 (67); The V-shaped tuberculum cerebelli is shown in gray, and the upper and lower rhombic lips by vertical and horizontal hatching, respectively. In c, open arrows show the migration paths from the rhombic lips. \textit{cbi} internal cerebellar bulge; \textit{ci} colliculus inferior; \textit{Cpb} corpus pontobulbare; \textit{cs} colliculus superior; \textit{is} isthmus; \textit{mes} mesencephalon; \textit{nV} trigeminal nerve; \textit{tbac} tuberculum acusticum; \textit{tcb} tuberculum cerebelli; \textit{tbpo} tuberculum ponto-olivare; 2, 4, 6: rhombomeres.

Important for cerebellar development is the isthmus organizer, a patterning centre at the midbrain-hindbrain boundary. Genes in this region encode transcription factors Otx2 and Gbx2. Otx2 is expressed in the midbrain and Gbx2 promotes development of the hindbrain through a mechanism involving transcription factors Wnt1 and Fgf8 (68). Precursors for different neuron types migrate to the their regions of destiny. Precursors to glutaminergic neurons originating from the rhombic lip differentiate under the influence of Math1, a transcription factor, into granule cells, unipolar brush cells and deep nuclear projection neurons (blue cell in Fig. 7, 68).
Granule neurons migrate tangentially to the surface of the cerebellum and generate the EGL. The EGL is a temporary structure that disappears as precursor cells from this region migrate inwards into the internal granular layer (IGL). This process ends in chicken about 10 days post hatching and in man up to 2 years after birth and eventually a three layered structure is reached from outward to inward comprising the molecular layer (ML), the Purkinje cell layer (PL) and the granular layer (Fig.8). Precursors to gabaergic neurons originating from the ventricular zone differentiate under the influence of the transcription factor Ptf1A into Purkinje cells, Golgi and Lugaro cells and deep nuclear interneurons (green cells in Fig.7). Differentiation of granule cell precursor into mature granule cell is regulated by Sonic hedgehog (Shh), a morphogen secreted by Purkinje cells (70, 71).

Purkinje cells migrate to a layer between the IGL and the molecular layer, which contains the expanding dendritic tree of Purkinje cells and their connections from granule cells and interneurons.
It is mostly unclear which factors promote differentiation of gabaergic neuronal precursors into Purkinje cells and other neuronal cell types. The hypothesis that inductive signals come from the rhombomere 1 roof plate awaits confirmation (73).

1.4.1 Apoptosis as a naturally occurring phenomenon in the developing cerebellum

Naturally occurring cell death is an integral part of normal development (74). Cell death by apoptosis was first defined by histological criteria of cell condensation, eosinophilic cytoplasm and condensation and fragmentation of the nucleus (75). The executioner proteins of apoptosis, the caspases, were first discovered in Caenorhabditis elegans. Pro- and anti-apoptotic proteins were also found in this model, and homologues were later identified in mammals. Apoptosis can also occur without involvement of effector caspases (76).

Apoptosis is initiated through an extrinsic or an intrinsic pathway (Fig. 9, 77). The extrinsic pathway involves death receptors like the tumor necrosis factor superfamily of
death receptors on the cell surface, including FAS, which on binding of a ligand forms a death-inducing signaling complex that activates caspase-8. Caspase-8 then activates other caspases, among them caspase-3, -6 and -7 that are responsible for inducing the ultrastructural changes in apoptosis. An array of other apoptotic stimuli, among them GCs and chemotherapeutic agents, leads to activation of the intrinsic pathway, which involves intracellular proapoptotic proteins like Bax and Bim. Bax forms pores in the mitochondrial membrane through which cytochrome c, one of the enzymes of the respiratory chain, can be released to the cytoplasm. Here it binds to Apaf-1 and caspase 9 in an apoptosome, which activates the common pathway of effector caspases 3, 6 and 7 resulting in microscopically visible morphological changes in the cell.

Figure 9. Apoptotic pathways according to Joza et al., 2002 (77)
The “extrinsic pathway” starts with the binding of ligand to a death receptor e.g. Fas. Fas then initiates recruitment of FADD and caspase-8 to the cytoplasmic regions of the receptor. Caspase-8 activates terminal caspases-3, -6 and -7. In response to endogen death stimuli e.g. GCs, distinct ‘BH3-only’ proteins, such as Bid and Bim, get activated in the “intrinsic pathway”. BH3-only proteins subsequently translocate to mitochondrial surfaces, where they associate with Bcl-2 protein. Anti-apoptotic Bcl-2 family members (e.g. Bcl-2, Bcl-XL) are inactivated, and pro-apoptotic Bcl-2 members potentially form mitochondrial pores or interact with pre-existing membrane channels. Release of cytochrome c (cyt c) induces formation of the apoptosome in association with Apaf1 and caspase 9. The apoptosome then activates effector caspases 3, 6 and 7. AIF induces hallmarks of apoptosis independent of caspases.
Apoptosis in the nervous system may serve different purposes like removal of non-functional cells, removal of neurons with erroneous connections and removal of superfluous cells (74). It is assumed that about 50% of all generated neurons in the CNS undergo apoptotic death in development. In several regions of the brain apoptosis occurs in two phases: first at the onset of neurogenesis and migration and later connected to synapse formation between differentiated neurons (78, 79). Studies of apoptosis in the cerebellum have concentrated on granule cells as their abundance and high developmental turnover allows the quantification of apoptotic profiles.

Both premigratory granule cells in the EGL and postmigratory cells in the IGL are affected by cell death (79, 55). Premigratory granule cells and neuronal progenitor cells undergo apoptotic death mainly because of the deficiency of morphogen Shh or growth factors like brain derived neurotrophic factor (70, 80). Postmigratory granule cells have been proposed to undergo apoptosis as a consequence of competition for synapse formation with other cells (80).

Purkinje cell death is estimated to occur in half of the originally generated neurons and occurs early in development during two phases in rodent cerebellum, around embryonic day 12 and directly postnatally (81, 82). Quantification of Purkinje cells after postnatal day 4 shows no further loss (83).

Genetic conditions, chemical substances and environmental factors can influence cerebellar development. Reduced cerebellar size and neuronal loss are associated with autosomal recessive ataxias and syndromes like Rett syndrome (X-linked deletion of methyl-CpG-binding protein 2, 84) in humans and mice. About 25 different genetic mutations in mice that cause cerebellar dysfunction including ataxia or neuronal loss have been described as harlequin, hoxc-8, lurcher, purkinje cell degeneration, and weebie mutants (www.informatics.jax.org). In other phenotypes like stumbler, meander tail and flathead the genetic defect is not defined yet.

Cerebellar apoptosis can be affected by different agents, e.g. ethanol (85), anticancer drugs e.g. cytosine arabinoside (AraC, 86), thyroid hormone (87, 88), and GCs (paper 2, 89).
1.4.2 Proliferation and differentiation in the developing cerebellum

Genes Pax 2, Wnt 1 and En1 regulate initialization of cerebellar growth. Pax 2, Wnt1 and En1 knockout mice do not develop a cerebellum at all (90), whereas transcription factor Math1 knock out mice do not develop an EGL (91). Other impairments in protein function like a dysfunctional retinoid-like orphan receptor α are known to reduce the external granule layer profoundly and a mutation in the gene coding for this receptor creates the characteristic phenotype staggerer (92). The granule cells of the vertebrate cerebellum proliferate during development until long after birth in all vertebrate species. The end of the proliferation period is visible by the disappearance of the EGL. Cells that arrest in G1 (Fig. 10) await various fates such as differentiation, apoptosis, quiescence (cells in this state do not enter the cell cycle, are smaller in size and have lower metabolism) and senescence (cells leave the cell cycle irreversibly), the latter two representing resting states of the cell. Differentiation often leads to an irreversible specialization of the cell.

Figure 10. Protein complexes of cell cycle regulator proteins called cyclins tightly regulate the cell cycle and cyclin-dependent kinases (CDK). Cells start replication in G1. In S phase chromosomes are synthesized such that a double chromosome set is generated. In G2 chromosomes migrate to their poles and in M phase the cells divides into to daughter cells (mitosis). During each of the four phases of the cell cycle a specific protein complex has to form in order for the cell to progress to the next phase (93).
The exact steps that lead to different cell types into different states are mostly unclear. To date some proteins have been defined that can promote differentiation. Cyclin-dependent kinase inhibitor p21\(^{\text{WAF1}}\) expression in the PC12 pheochromocytoma cell line leads to differentiation of PC12-cell into neurons. Cyclin-dependent kinase inhibitors might present a link to the mechanisms that promote neuronal differentiation by arresting cells in G1 phase (94). Mice with a deletion of another cyclin-dependent kinase inhibitor p27 have larger cerebella and extended cell cycle in the EGL. P27 is specifically expressed in cells within the inner EGL (95) indicating that it might have a role in proliferation control and differentiation for granule neurons. Interestingly, GCs have been found to upregulate p27 translation in non-neuronal cell types like breast cancer cells (96), osteoblasts (97) and stimulated lymphocytes (98).

During the development of the cerebellum granule cells replicate intensely. Purkinje cells secrete Shh, a ligand to the receptor Patched. Patched is located in the cell membrane of granule cells (70). On ligand binding, Patched will promote expression of genes like Nmyc and Cyclin D1, which leads to replication of the cell. Shh can prevent death of cerebellar granule cells induced by HC but not when death is induced by Dex by upregulating 11β-HSD2 (99). Other growth factors e.g. insulin-like growth factor 1 (100), basic fibroblast growth factor (101) or brain-derived neuronal growth factor (102) are known to increase proliferation in the cerebellar neuronal population.

Lately another protein called REN has gained attention with respect to regulation of proliferation and differentiation. The deletion of this gene was discovered in medulloblastoma, which is a tumor originating in immature granule cells of the cerebellum (103). In further studies it was shown that REN is expressed in granule neurons on the inner rim of the EGL and in the IGL. Overexpression leads to reduced Bromdeoxyruridine (BrdU) incorporation and increased Neuronal Nuclei (NeuN) protein expression in cultured granule neurons thus showing promotion of differentiation (104). REN could also be shown to counteract the Shh pathway.

Changes in cell proliferation and differentiation in the cerebellum are associated with syndromes like the different autosomal recessive ataxias and Rett syndrome in humans and mice (X-linked deletion of methyl-CdP-binding protein 2, 84). Different agents like ethanol, nicotine (105), and thyroid hormone (106) can also disturb birth and maturation of granule cells and other neurons.
1.5 The cerebellum and its role in motor control and higher brain functions

The cerebellum is through the inferior, middle and superior peduncle connected to different parts of the spinal cord and olive, vestibular organ, motor cortex, dorsal prefrontal areas and extrapyramidal system (107). Holmes has described characteristic symptoms of voluntary and involuntary motor control as a consequence of cerebellar damage in man as early as in 1939 (108). They comprise abnormalities in the rate, regularity and force of voluntary movements, postural hypotonia, and a mild degree of asthenia and fatigability of the muscles. Others have shown that ablation of the cerebellum leads to loss of learned motor sequences (109). Later disordered eye movements (nystagmus), poor articulation (dysarthria), impaired swallowing and tremor were also recognized as symptoms of cerebellar affection (110).

It is striking that only a fraction of the total cerebellar area is occupied with motor control. It has been proposed that other regions may be involved in control of higher brain functions. Indeed, functional imaging has shown that some cerebellar lesions give rise to impairment of verbal working memory (the ability to name a list of digits backwards after they have been named, 111, 112).

The concept of a “cerebellar cognitive affective syndrome” proposed by Schmahmann et al. (110) is characterized by disturbances of executive function (deficient planning and abstract reasoning), impaired spatial cognition, personality change (flattening of affect), and linguistic difficulties (dysprosodia, agrammatism) both in adults and children with cerebellar damage of variable origin. The connection of the cerebellum with dorsal prefrontal areas as responsible for planning activities may represent the anatomic correlate to disturbances in higher brain functions, especially executive functions (107).

Interestingly, postmortem analysis of cerebellar changes in autism cases has reported cerebellar changes in 54%, predominantly flocculonodular dysplasia, thus linking the cerebellum to autism (113).

1.6 Nuclear receptors (NRs)

Effects of hormones on DNA were first noted by Clever et al. (114), who experimented with the insect steroid hormone ecdysone and saw changes on the giant chromosomes of *Chironomus tentans*, a non-biting midge, upon treatment. The human GR and the
estrogen receptor (ER) were the first NRs to be cloned and expressed in 1985 (115, 116). Subsequent sequencing of NRs demonstrated the general structural organization principle of the NR (Fig. 11, 117)

NRs have a variable N-terminal modulator region, a well conserved central domain and a relatively well conserved C-terminal ligand-binding domain. The central domain forms structural motifs called zinc fingers (zinc ions bind to cysteine and histidine and thereby stabilize polypeptide loops) accounting for DNA binding. Ligand binds to the C-terminal region. For some receptors (orphan receptor like nerve growth factor induced clone B/NR4A1) the ligand is unknown. Upon translocation to the nucleus NRs bind to responsive elements in the promoter regions of target genes. Consensus sequences have been published for most nuclear receptors except mineral corticoid receptor (MR, 117). With new identification techniques 150 base sequence variants could be identified for the Glucocorticoid responsive element (GRE) alone 118). In addition, responsive elements are not strictly possessed by one receptor type. Mineralcorticoid receptor (MR) or the progesterone receptor can for example bind to the GRE. Responsive elements can induce and reduce expression of a target gene. The contrary to the “positive” GRE is the “negative” mouse mammary tumor virus hormone responsive element (MMTV-HRE), which reduces expression of target genes.

An evolutionary-based nomenclature of the NR superfamily was published in 1999 (119). It consists of 6 evolutionary closely related subgroups (NR1-6). NRs have been found in most metazoans (multicellular animals with differentiated tissues) and gene sequences and protein structures are markedly conserved.
1.6.1 Glucocorticoid receptor (NR3C1) and its ligands

The discovery of GC and the GR started with the study of hormones from organ extracts (for review see 120). Later on the connection between stress and amelioration in autoimmune disease or allergy was recognized and lead to the first clinical application of Cort in 1948 (24). After this initial trial the pharmaceutical industry developed many different substances related to the original steroid hormone purified from organ extract (121). Prominent members of the first generation of GCs are prednisolone and Dex. With treatment trials a range of GC side effects on different organs emerged (see also 1.3), among these changes in glucose and insulin level, bone, fluid and electrolyte balance and CNS symptoms like depression.

Radioactively labelled GCs could now also be used to define binding sites in cells. One of the earliest reports describes nuclear binding sites in rat thymus cells (122). GR is found in most cells types of the body. The GR was first cloned in 1985 and found to exist in two alternatively spliced transcripts: hGRα and hGRβ (Fig. 12, 115). Whereas the hGRα acts as a classical ligand operated nuclear receptor, hGRβ does not interact with GCs or bind to GRE. It has a varied expression in different mammalians, tissues, and cell types in the same tissue. Cotransfection with hGRα revealed a dominant negative effect on GR action, thereby connecting the expression level of hGRβ to steroid resistance (123).

Figure 12. Structure of the hGR according to Zhou et al. 2005 (123).

The N-terminus represents the constitutive transcriptional activation function 1 (AF1), while the C-terminus encodes the ligand-binding domain (LBD) and ligand-dependent activation function 2 (AF2). The highly conserved DNA-binding domain (DBD) is located in the central region of the protein. In addition, the domains involved in nuclear localization, receptor dimerization, and cofactor binding are mainly localized to the C-terminal ligand-binding motif. H hinge region.
GR forms an oligomer with heat shock protein 90 (hsp90), hsp70, p23, and hsp90-binding tetratricopeptide repeat protein in its resting state in the cytoplasm. Upon binding of ligand, hsp 70 dissociates and GR initiates transport to the nucleus through a rapid hsp90 dependent and a slower hsp90 independent mechanism (the latter thought to be mainly diffusion, 124). Hsp90 stays in the complex with GR on the way from the cytoplasm to the nucleus and in the nucleus itself. It is suggested that it might even be important for relocation of GR to the cytoplasm (124). Hsp90 also facilitates binding to the microtubules and is especially important for long distance transport in neurites of neurons (125). Other molecules like importins also play a role in nuclear import and export of the GR (for review see 125).

A steroid ligand may bind both to GR and MR, but with different affinity (Fig. 13). The mineralcorticoid aldosterone binds with high affinity to MR and with lower affinity to GR (Fig. 10, 126).

![Figure 13. Affinity of different steroid ligands to MR and GR respectively according to Rupprecht et al., 1993 (126)](image)

Binding affinity (Kᵢ values determined from the displacement curves) vs. functional sensitivity (ED₅₀) in the co-transfection assay for human mineralocorticoid (left panel) and human glucocorticoid receptor (right panel). In order to estimate ED₅₀ values SK-N-MC cells were cotransfected with MTV-LUC (mammary tumor virus luciferase response element) and either human mineralocorticoid receptor or human glucocorticoid receptor. The induction of the MTV-promoter was calculated from the relative light units measured in the individual cell extracts with or without ligand treatment. The baseline activity of the MTV promoter without addition of hormone, equivalent to approximately 2000 RLU, was set at 1. On the resulting dose response curves the functional sensitivity (ED₅₀) was estimated.

The GR ligand’s affinity and ability to affect gene transcription correlates with its “relative steroid potency” (127), which is measured by effects on the HPA axis, glycogen
deposition, and anti-inflammatory and eosinopenic effects. Recently, evidence has emerged that suggests that some aspects of GC effect like induction of cell death might not correlate with “relative steroid potency” (paper 1, 128), suggesting alternative steroid pathways or effects on different receptors.

Receptor agonists and antagonists provide a useful tool for the study of hormone-induced effects. The development of the selective GR agonist RU28362 and the mineral corticoid receptor antagonist RU28318 made it possible to dissect the effects of the GR and MR in different tissues. The competitive GR antagonist Mifepristone or RU38486 was developed as an abortion drug. It has strong anti-glucocorticoid and anti-progesterone effects coinciding with a high affinity to the GR and PR (129). Notably, in some species like chicken RU486 does not react with the PR but only with the GR (130).

1.6.1.1 GR and its ligands in the immature and mature CNS

Although GC administration is mainly targeting GRs outside the CNS, side effects on the brain have been noted early on (24).

Studies in the rat brain show that GR expression is distributed over the whole brain with high density in the olfactory cortex, hippocampal formation, amygdala, hypothalamus and cerebellum (131). A study in newborn mice found that GR is expressed in highest amount in the neonatal cerebellum (132). In contrast, MR is not expressed in detectable levels in cerebellum, but has its highest expression in the hippocampus. Notably, the MR in the CNS does not show mineral corticoid selectivity and will bind e.g. Cort with higher affinity than the GR (133). Thereby Cort, the endogen steroid in birds and rodents, or HC, the endogen steroid in dogs and primates, can occupy all of the MR in the brain at low basal levels. In order to exert GR mediated effects in the brain levels of naturally occurring, steroid have to be high e.g. with stress.

The access of glucocorticoids to the brain is dependent on several factors, among these binding to the corticosteroid-binding globulin (CBG) in blood, local metabolic conversion of steroids influenced by 11β-hydroxysteroid dehydrogenases, and the blood-brain barrier (BBB).

About 95 % of circulating Cort and 75 % of HC are bound to CBG (HC binds in ca 20 % to serum albumin) thus only leaving 5 % as biologically active. Dex has a low affinity for CBG and is mostly bound to albumin. Plasma protein binding for Dex has been reported
to be 68-75% (134). Stress downregulates CBG thereby increasing the availability of free Cort and HC (135).

Next, steroids must cross the BBB in order to exert their effect in the brain tissue. Dex and other high potency steroids are more lipid soluble agents than naturally occurring steroids, and thus could cross the BBB more readily. In addition the BBB function may be reduced in premature infants (136). On the other hand the ABC transporter protein Mdr1a expressed in endothelial cells of the BBB can specifically prevent synthetic steroids from passing the barrier (137). But subsequent studies in pregnant guinea pigs showed that Dex, given to mothers, dose dependently reduces CRH mRNA in the fetus, thus indicating that synthetic GCs indeed pass the BBB in appreciable amounts in immature organisms (138) probably most likely because of the diminished barrier function in premature infants.

When GCs finally have crossed the BBB, there are other factors that influence GC effect. Among these the enzyme 11-beta-hydroxysteroid-dehydrogenase type 2 (11β-HSD2) has a role as inactivator of Cort. It is expressed in the developing CNS, including cerebellar granule neurons (139) and blocks endogenous GC effects.

1.6.1.2 Non-genomic actions of GR

Early reports of rapid or non-classical actions of steroids described instant anesthetic effects of injected progesterone in rats (140) and rapid action of aldosterone (141). The common denominator was the short time of action that was not compatible with the classic genomic action of steroids. In recent years rapid signalling for almost all steroid hormones has been discovered (142). In 2003 the Mannheim classification categorized non-genomic steroid actions into two main types: direct and indirect, meaning that steroid hormone acts alone or with a co-agonist, respectively (143). For further specification see fig. 14. The non-classical steroid action can principally be signalled either by the classical steroid nuclear receptor or by a non-classical e.g. membrane bound receptor.
Also for all other steroid ligands the existence of membrane-bound classical or non-classical receptors has been proposed (for review see 143). Examples of rapid non-genomic action in the CNS involved serotonergic responses in the limbic system of lizards 20 min after injection of Cort (144), transient increase of aspartate and glutamate levels in the CA1 area of the hippocampus 15 min after injection of Cort (145) and increase of Na⁺-dependent uptake of glutamate in rat cerebral cortex synaptosomes 15 min after injection of Dex (146). Behavioural changes as a possible consequence of biochemical changes in the CNS could also be demonstrated. Suppression of courtship behaviour by stress could be mimicked by injection of Cort in the amphibian *Taricha granulosa* already 8 minutes after injection (147).

Membrane-bound GR may have a role in programmed cell death. Its involvement has been demonstrated for the apoptosis of human lymphocytes in the CCRF-CEM cell line (148). Since Dex killed cerebellar granule neurons in rats rapidly and the NMDA receptor antagonist MK801 blocked this action, a cross talk between the NMDA receptor and an either membrane-bound or cytosolic GR has been proposed (for more detailed discussion of GR/NMDA interaction see paper 1 and 2).
1.6.2 NR4 group

The first member of this group, nerve growth factor induced clone B (NGFI-B), was described in 1988 (149). All members (NGFI-B or NR4A1, Nurr1 or NR4A2, NOR1 or NR4A3) are so-called “orphan” receptors meaning that they have no known ligand. A hydrophobic cleft as ligand binding site for steroid hormones has not been found in these receptors. NGFI-B and the other members of the NR4 group were eventually found to be immediate early genes, which are activated rapidly, transiently and independently of protein synthesis by multiple stimuli including phorbol esters, growth factors, calcium ionophores and membrane depolarization (150, 151).

The receptors can bind to a specific response element called NGFI-B response element (NBRE) and a palindromic version of NBRE called NurRE. Homodimers bind preferentially to NuRE. Heterodimers, which e.g. contain retinoid X receptor, bind preferentially to several sequences of NBRE (152, 153, 154, 155, 156). The target genes of the NR4 group are largely unknown (157) but the NR4 group plays a role in the development of dopaminergic neurons in the midbrain and in regulation of the HPA axis of steroid synthesis (see 1.6.2.1) as well as apoptosis.

The members of the NR4A family have as yet not been cloned in chicken, but homologues of NGFI-B are found in several species, including rat (NGFI-B), mouse (nur77), and human (TR3), as well as non-mammalian model organisms such as *Xenopus laevis* (158), and the zebra fish *Danio rerio*. NR4A2 and NR4A3 homologues are also found in both zebra fish and *Xenopus*.

1.6.2.1 NR4 in the CNS

Both NGFI-B and Nurr 1 are expressed in mouse brain with variations depending on the developmental stage. Nurr1 could be detected early on in the embryo while NGFI-B first was detected postnatally. Both are also expressed in cerebellum (159). NOR1 was detected only in low levels in the brain. No obvious differences of development were shown in knockout mouse models of NGFI-B (160) whereas knockout models for Nurr1 died 12 hours after birth. Analysis revealed that Nurr1 is essential for the survival of dopaminergic neurons in the midbrain that are lost in Parkinson disease (161).

Another role for Nurr 1 and NGFI-B emerged from study of the regulation of the HPA axis (see 1.1.3) of steroid synthesis (162). CRH, produced in the paraventricular nucleus
of the hypothalamus, induces increase of POMC, a precursor of ACTH in the anterior pituitary gland. ACTH in turn increases production of steroid hormones in the adrenal gland. Stress induces Nurr 1 and NGFI-B expression in the paraventricular nucleus (CRH production) and in the anterior pituitary. Nurr 1 could be shown to enhance transcription of CRH in the hypothalamus and ACTH in the anterior pituitary gland. In contrast GCs decrease transcription through a negative GRE in the promoter regions of CRH and ACTH. Thus Nurr1 has an upregulating effect on steroid hormones. The evidence for GR and NR4A group crosstalk is discussed in 5.2.2.
2. Aims of the thesis

The aims of the thesis have been to study the influence of glucocorticoid hormones on the immature central nervous system.

Subgoals:
- To establish the chicken embryo cerebellum as a simple model system and chicken embryonic cerebellar neuron culture for the evaluation of hormonal effects on immature neurons.
- To investigate the effect of GCs on granule cell proliferation, cell death and differentiation in chicken embryo cerebellum and in cerebellar neuron culture.
- To establish a new method for gene transfection of chicken cerebellar granule neuron (CCGN) cultures for the study of GR regulation.
3. Methods

3.1. Chicken embryo cerebellum model

This thesis presents the chicken embryo cerebellum as a model system to test neurotoxic effects of drug exposure in early life. The chicken embryo is a classical model organism for developmental and neurobiological studies (163). Lately the whole chicken genome has been sequenced and a 75% similarity to the human genome was found. Differences include the lack of gene families related to egg laying in the human genome (163). The chicken embryo represents a practical and low cost animal model, which reproduces quickly and does not require animal facilities. The maturation of the chick embryo is only dependent on the correct temperature and humidity during incubation. The yolk sac contains initially the maternally derived steroid hormone Cort, though not HC. Cort, the main GC in birds, had a concentration of 1,8 ng/ml (5.2 μM/l) on ED16 in the embryo and increased to 2,3 ng/ml (6.6 μM/l) before hatching (164). Thus basal levels of endogenous Cort are low, and effects observed with GC treatment would mainly be a consequence of administered GCs. The chick GR has been cloned and has a homology of 73% with the human GR (165).

Administration of GCs through the eggshell resulted in changes of chicken neuroendocrinological tissue in earlier studies (166). Jochemsen et al. (167) showed that BrdU could be detected in various chicken organs 24 hours after injection, though not in all embryos (167).

The histological architecture of the cerebellum is markedly conserved in all vertebrates. The adult vertebrate cerebellum has three layers (66) comprising the ML, the PL and the IGL. During development the EGL appears as a temporary structure (see section 1.5), which in man exists up to 2 years after birth and in birds until about 9-10 days after hatching. Main cell types are easily identifiable in the different layers.

Based on histological criteria like EGL thickness developmental stages of the animal model cerebellum can be compared to stages in the human cerebellum (55). The chicken cerebellum on E17 (paper 2 and 4) has a relatively thick EGL, at least a bilayer of Purkinje cells and no apparent glial fibrillary acid protein expression, thus resembling the human cerebellar cortex in late 2nd to early 3rd trimester, i.e. about 24 to 32 gestational weeks (168, 55).
3.2 Chicken cerebellar granule neuron (CCGN) culture

Rat cerebellar granule neuron cultures have been extensively used for studies of excitotoxicity and apoptosis (150, 89), but our group first established chicken cerebellar granule neuron cultures in 2006 (paper 1). The combination of the in vivo model with cell culture offers possibilities for the confirmation of results and the in depth study of mechanisms by transferring observed phenomena from the in vivo model to the more easily manipulated cell culture. Thus, GC effects on apoptosis, proliferation and differentiation of granule neurons in GC treated cerebella could be confirmed in cell culture (paper 2 and 4). Purified granule cell cultures can be treated with agonists and antagonists of intracellular signalling pathways (paper 1, 2 and 4).

Chicken cerebellar granule cell cultures (CCGNs) are also suitable for transfection techniques. CCGNs can be transfected with a plasmid encoding the protein of interest and by different transfection techniques suitable for biochemical and microscopic studies (paper 3).

The cerebellar neuron culture is an artificial cell system. Neurons do not connect properly with their target cells and axonal-dendritic orientation is disturbed. CCGNs seem mainly to derive from the EGL (169) thus representing the immature granule precursor population. On day 3 though, neurons show expression of glutamate receptors (paper 1) and NeuN (paper 4) indicating a differentiation process. A low dose of the cytostatic agent AraC was used to prevent overgrowth of astrocytes. Under these conditions a purity of 80% CCGNs was achieved in culture.

The use of chicken serum (10%), which is not analyzed in all its components, implies that interaction with unknown growth and survival factors cannot be ruled out, and may influence reproducibility of results of experiments in culture.

When compared to cultured cerebellar granule neurons from young rats, a well-known model for the study of excitotoxicity (170), CCGNs had the advantage that they could be grown in 5mM K+. Thus, CCGNs could be cultured under more physiological conditions than rat neurons, which demand depolarizing conditions (25mM K+, 171). It could also be shown that CCGNs mature earlier in culture than rat neurons since excitotoxicity was induced earlier in chicken culture (3 days in vitro, DIV) compared to rat (7 DIV, paper 1).
3.3 Methods for the detection of apoptosis

Cerebellar slices were stained with hematoxylin and eosin (HE) for histological identification and quantification of apoptotic cells. The cytoplasm of apoptotic cells becomes eosinophilic and cells and nuclei condense, resulting in a distinct cell morphology (paper 2). These morphological changes still represent the gold standard for the detection of apoptosis. Caspase-3 expression, frequently used for the detection of apoptotic death, showed a similar dose-dependent GC effect. Caspase-3 expression gives an indication of apoptotic death, but apoptosis can also be a caspase-independent process and caspases are not only activated in other processes than apoptosis (172). Thus, in addition to caspase-3 immunohistochemistry other methods of apoptosis detection have to be used.

For the detection of apoptotic death in CCGN culture (paper 2) a combination of four different techniques was used. Firstly, the trypan blue exclusion assay is based on cell membrane impermeability for trypan blue in living cells. The trypan blue assay in combination with phase contrast allows for identification of the cell type of the dead cell. But the assay cannot distinguish necrotic and apoptotic cells. Secondly, caspase-3 immunohistochemistry was used for determining apoptosis. The amount of caspase-3 positive cells in culture was lower than the amount of trypan blue including cells, because the trypan blue assay includes all dead cells independent of cell death mechanism. Decrease of nuclear diameter represents another typical feature of apoptosis (173), which was applied to rule out false-positive caspase-activation 172. As a fourth method, caspase-3-dependent cell death was confirmed by using caspase-3-inhibitor to block GC induced cell death on cultured neurons.

Quantification of cell death can be problematic in model systems since apoptotic cells are removed rapidly from the system by phagocytosis (75, 174). In the chicken cerebellum peak apoptotic cell death is reached around E16 (unpublished results). Thus, a high turnover of immature neurons occurs in a relative short time span. This allows apoptotic cells in the chicken cerebellum to be quantified both at basal rates and increased rates after drug exposure (paper 2).
3.4 Methods for the detection of proliferation and differentiation

BrdU incorporation is a common method to measure cell proliferation in vivo (paper 4). The antibody is directed against BrdU in the nucleus and dividing cells incorporating BrdU are stained. BrdU can be detected in cells several days after cell division (169). BrdU was injected 3 hours before sacrifice in this study (paper 4). Thereby most likely cells in S-phase were marked, as the S-phase of the cell cycle in neurons lasts about 3 hours (175). In cultures proliferating cell nuclear antigen (PCNA) was used, which is expressed by proliferating cells in the S-phase (paper 4). NeuN and microtubule-associated protein 2 (Map2) were used as differentiation markers of postmitotic neurons (paper 4, 176, 177, 178).

3.5 Transfection of CCGN

The transfection of neurons has generally proven to be difficult. While transfection using liposomes is a well-known transfection technique for adherent cells, electroporation has traditionally been used for transfection of cells in suspension. The development of instruments such as the Cellaxess® CX1 system also made the latter method applicable to adherent cells (paper 3). In paper 3 two transfection methods were compared and optimized for use in CCGN. The relatively high amount of DNA used for transfection represents one of the disadvantages of the electroporation method. Also, this method is based on a strong electrical current that leads to a disruption of the cell membrane, which in turn allow plasmids to enter the cell (179). It can be assumed that neurons in the whole culture dish will be exposed to a certain degree of electrical shock and this can have the potential of changing cell culture properties. Such electrical impulses as given during the electroporation procedure have been shown to profoundly alter the genetic profile, especially the expression of growth factors in neuronal tissues (180, 181, 182) and consequently also biological processes such as apoptosis and proliferation can be affected (182). Further investigations into these side effects of electroporation on CCGN culture are required. In our studies the liposome method was most suitable for biochemical studies and was used for measuring GR mediated gene transcription by Dex and HC (paper 2) and the interaction between GR and NGFI-B (paper 3). On the other hand, the electroporation method was used for protein localization studies (paper 3).
4. Summary of papers

Paper 1

This paper describes how CCGNs are prepared and how such cultures can be use as a model for detecting neuronal damage by diverse agents. An 80% enriched neuronal culture was achieved by adapting the established method of preparing rat cerebellar neuronal culture and by using chicken serum instead of fetal bovine serum in the culture medium. Chicken cultures developed more rapidly than rat neuron cultures as indicated by earlier neurotoxic reaction to glutamate. Unexpectedly, CCGNs could also be grown in medium containing 5 mM K⁺ (physiological concentration) without significant change in survival in contrast to rat cerebellar neurons, which demanded 25 mM K⁺ in the culture medium. When assessed for cell death mechanisms CCGN showed similar production of reactive oxygen species (ROS) measured by dihydroethidium oxidation, similar level of caspase-3 activation measured by caspase-3 assay and immunofluorescent staining of expressed caspase-3 protein as rat cultures.

The CCGN culture therefore provides a useful, low-cost and reproducible method for studying neurotoxic mechanisms and holds certain advantages over rat cultures such as more physiological cell culture conditions.

Paper 2

The effects of the high potency steroid Dex and the low potency steroid HC on cell survival were evaluated in chicken embryo cerebellum and CCGN culture. Dex and HC induced granule cell death in the IGL of the chicken cerebellar cortex and in culture in a dose dependent manner. The mode of GC induced cell death was shown to be apoptosis. Cells showed typical apoptotic morphology and activation of caspase-3 in chicken embryo and CCGN culture. Caspase-3 inhibitor treatment of cerebellar cell cultures before GC treatment abolished cell death. Interestingly, cell death induced by Dex and HC could be blocked by pretreatment with mineralcorticoid antagonist RU 28318 and NMDA receptor antagonist MK801, indicating a possible crosstalk between a membrane-bound GR and NMDA receptors. Maximum effect on gene transcription for Dex and HC was evaluated by transfection of cerebellar neuron culture with a GRE3-Luc reporter gene. Dex and HC showed high transcription activity at 0,01nM and at 10nM, respectively. Thus, HC concentration needed to induce transcription of target genes was
much closer to the dosage causing neurotoxic effects on neurons (1μM) giving HC a less favourable treatment profile in vitro when assessing apoptosis.

**Paper 3**

Two different methods were optimized for transfection of CCGNs. The electroporation method using the Cellaxess® system yielded transfected neurons in a local well-defined region of the culture dish thus facilitating microscopic studies of tagged target proteins. The liposome based method using Metafectene® Pro generated transfected cells evenly distributed over the whole culture dish and was therefore more suited for biochemical studies. The liposome method was subsequently used to study the interaction of GR with NGFI-B. CCGNs were transfected with the reporter gene NBRE8-luc and then treated with forskolin and PMA. Relative luciferase activity increased indicating the induction of the endogenous NR (nuclear receptor) 4A family in CCGNs. As forskolin and PMA unselectively activate a range of other factors, NGFI-B was chosen to be overexpressed in CCGNs. Cotransfection with GR reporter gene GRE3-luc revealed that NGFI-B in its wild type significantly downregulated ligand (Dex) induced reporter gene activation. A C-terminal truncated version of NGFI-B downregulated ligand (Dex) induced reporter gene activation non-significantly and much less than wild type NGFI-B, indicating that parts of the C-terminal of NGFI-B are involved in crosstalk with the GR.

**Paper 4**

This paper addressed GC effects on both proliferation and differentiation in chicken embryo cerebellum and cell culture. Dex and HC significantly reduced the number of proliferating neurons in a dose dependent manner in the IGL of the chicken cerebellum. Decreased proliferation appeared to involve the GR, as GR antagonist RU 38486 could antagonize this reduction. Dex and HC increased the number of NeuN positive cells in the IGL and expression of Map2 in whole cerebellum homogenated lysate. Apart from inducing cell death the antimetabolite AraC is also known to arrest cells in G1-phase. AraC significantly increased the number of NeuN positive neurons in culture similar to Dex thereby indicating that arrest of cells in G1 phase may be sufficient to influence differentiation.
5. General discussion

5.1 GC effect on apoptosis, proliferation and differentiation in the immature cerebellum

Our observation of GC induced apoptosis in the chicken embryo cerebellum and in CCGN (paper 2) is in accordance with studies in other species (99), and similar results have also been obtained in other brain regions (51, 183, 184). We compared HC to Dex and found it to be equally neurotoxic than Dex (paper 2). Other studies have used Dex (51, 183, 184) but not HC. In the clinical situation though, Dex seems to be more neurotoxic when used for prophylaxis of BPD compared to HC (see 5.4). Different factors might account for this difference. Since Dex is more lipophilic than HC Dex may more easily cross cell membranes. The ABC transporter protein Mdr1a expressed in the cells of the BBB prevents synthetic steroids from passing the barrier (133). But preterm babies are known to have a reduced function of the BBB. As a result, Dex might be more neurotoxic in humans, although HC shows equal neurotoxicity in our in vivo model. Alternatively, other GC effects may be more important, such as effects on proliferation and differentiation.

It has been discussed if MR could signal GC action. Although this might be the case in the hippocampus region, MR has not been detected in the fetal cerebellum (185, 186). The protective effect of MR antagonist RU28318 may be due to its antagonistic effect on the GR (paper 2, 187).

GCs inhibited neuronal proliferation in vivo in accordance with other studies in the cerebellum (paper 4, 54, 99, and other brain regions 188, 189, 190). We found that Dex, compared to HC, leads to inhibitory effects on proliferation at lower concentrations, which represent the therapeutically range in the clinical situation (paper 4). In CCGN Dex (but not HC) showed a significant antiproliferative effect. Other studies have either used Dex (189, 190), corticosterone (188) or both in combination (99), or HC in high dosage (10mg/kg, 54). If proliferation is inhibited, the final number of functioning neurons in the cerebellum will necessarily be reduced. If the reduction is considerable, it will manifest itself as smaller cerebellar cortex volumes and secondarily reduced white matter volumes. This has been shown for Dex treatment in preterm infants (191) but not for HC (192).
Dex was shown to have a stronger in vivo and in vitro effect on granule cell proliferation than HC (paper 2) whereas the effect on apoptosis was more similar occurring at high doses for the two drugs (paper 4). There might, however, also be a direct connection between proliferation and apoptosis. It was shown that low dose Dex induced apoptotic death of premitotic neurons in the EGL (55). This points to an indirect effect of low dose Dex on proliferation rates in the IGL via induction of apoptosis in the EGL.

In conclusion Dex has significant effects on proliferation and apoptosis in vivo and in vitro, whereas HC has significant effects on apoptosis in vivo and in vitro but only a moderate effect on proliferation in vivo. This evidence might be reflected in clinical results of increased major neurological impairment with Dex treatment but not with HC treatment (40).

We observed increased neuronal differentiation after GC treatment in chicken cerebellum and neuronal cell culture (paper 4). Few other publications have studied this effect. In mice pups treated with Dex (99) a higher expression of the differentiation marker Zic1 was found in whole cell lysates of Dex treated animals compared to controls. In contrast, in other brain regions such as adult rat hippocampus (190) and newborn primate hippocampus (63) GCs did not increase differentiation. Arrest of cells in the G1 phase of the cell cycle as a result of GC treatment (54) may represent the signal for neuronal differentiation (paper 4). Thus, increased neuronal differentiation may be a consequence of reduced proliferation caused by GC treatment.

5.3 Mechanisms of GC action in CCGN

5.3.1 Classical versus non-classical GC action

Principally GC action can be classified in classical and non-classical (see chapter 1.7.1.2). The classical steroid action involves the cytoplasmatic receptor and transcription of target genes. Non-classical actions involve second messengers in their signaling pathways and can either be executed via the classical steroid receptor or a putative membrane-associated GR. The membrane binding form of GR is characterized by high affinity for Cort and HC, and low affinity for aldosterone, Dex and RU 38486, whereas RU 38486 has high affinity to the cytoplasmatic GR receptor followed by Dex, Cort and aldosterone (193). In paper 2 the apoptotic effect of Dex and HC seem to involve a non-genomic steroid action, supported by the fact that a comparatively high GC dose was
needed to result in significant apoptotic effects in vivo and in vitro. The effect was rapid as shown in another study in rat (89). Dex-induced cell death could be blocked by MK801, a NMDA antagonist, thereby indicating a cross talk between the NMDA receptor and a membrane bound GR (paper 2, 89). Possible crosstalk between the GR and the NMDA receptor has also been suggested in other studies (194, 195).

A relatively low dose of Dex compared to HC significantly reduced proliferation and induced differentiation in the chicken cerebellum (0.1mg and 5 mg/kg egg respectively, paper 4). This reflects the difference in relative steroid potency between Dex and HC. Also, the antiproliferative effect of GCs could significantly be blocked by GC antagonist RU 38486 (paper 4). RU 38486 has a high affinity to the cytoplasmatic form of the GR. Together this evidence argues for a classical steroid effect on proliferation and differentiation. Notably, RU 38486 reacts in chicken only with GR and not with PR (130).

5.3.2 Interaction of GR with the nuclear receptor group NR4 in neurons

Early reports about possible interactions between GR and the NR4A group came from studies in adult rat hippocampus (196). Here it could be shown that GR mRNA was increased and NGFI-B mRNA reduced by placing the animals in an enriched environment and measured by in situ hybridization. Philips et al. (197) demonstrated the downregulation of the CRH induced transcription of NGFI-B mRNA in a mouse pituitary epithelial-like tumor cell line (AtT-20). Reduced transcription of CRH as a result of GC interaction with a negative GRE in the CRH promoter (198) could account for consecutively reduced transcription of NGFI-B. Furthermore GR-induced repression of POMC transcription happened at the NuRE in the promoter region of POMC, but not in a binding fashion to the response element but rather a protein-protein interaction between GR and NGFI-B and/or other cofactors. NuRE is a palindromic NBRE$_3$ sequence, which binds preferably dimers with at least one molecule of NGFI-B (199). Interaction between NGFI-B and GR was shown in cells with both proteins overexpressed. In our study co-transfected chicken cerebellar neurons with overexpressed wild type NGFI-B and NBRE$_8$luc showed no downregulation of NGFI-B-induced gene transcription upon treatment with Dex, which activates the endogenous GR (paper 4). The construction of the artificial response element with 8 copies of the response sequence might make a protein-protein interaction of endogen GR and NGFI-B at the response element less
efficient. Also, overexpression of NGFI-B could give such a strong signal that it cannot be overcome by endogenous ligand-activated GR. In addition, protein-protein interaction could be hampered by a different co-factor profile in chicken cerebellar neurons. Philips et al. (197) and Martens et al. (199) try to define the domain of the GR and NGFI-B, respectively, which interact when mutually blocking gene transcription. In both the GR and NGFI-B the DNA-binding domain (DBD) was hypothesized to be dominant in blocking interaction although the deletion of the C-terminus in GR led to ligand-independent blocking (197). The negative control (deleting the DBD) could not be performed because this would compromise the reporter gene assay, as the readout is dependent on DBD binding. The C-terminal deleted NGFI-B construct (Δ 352) showed a slight reduction in blocking the GR/Dex induced GRE-luc activity in contrast to Δ 380 construct which shows full blocking activity. The construct used in paper 3 is a Δ 369-597 construct of NGFI-B, which lies between the two constructs used of Philips et al. concerning its starting point of C-terminal deletion. There was a tendency that NGFI-Btr blocked GRE-luc activity to a lesser degree than wild type NGFI-B (paper 4). These results suggest that not only the DBD region of NGFI-B is involved in protein-protein interaction with GR or a common cofactor (197) but also parts of the C-terminus itself. In difference to studies in non-neuronal cell lines (199, 197) paper 3 shows GR and NGFI-B interaction in primary cerebellar neuron cultures thereby confirming the relevance of this interaction in the CNS.

5.4 Possible clinical implications

It has been suggested that HC might be a potentially safer GC in prophylactic treatment of preterm babies for BPD. Our results support this hypothesis. When the effects on apoptosis and proliferation are taken together, HC had a less neurotoxic effect in our model compared with Dex (paper 2 and 4). The evaluation of HC as the drug of choice for prevention of BPD, however, has to be based on its effects on BPD and neurodevelopmental outcome compared to other treatment alternatives such as Dex. A meta-analysis of treatment effects in different randomized clinical studies has estimated a risk ratio of 0.97 (test for overall effect p=0.37) indicating that treatment with HC does not change BPD outcome (fig. 4A, introduction, 40) whereas Dex reduces the risk of BPD significantly (risk ratio 0.73, test for overall effect p=0.00001).
Several studies addressed the long-term neurodevelopmental consequences of HC using different approaches, such as neurological examination, the WISCII test for IQ, the ABC test for motor development and MRI brain scanning for structural changes (200, 201, 202, 203, 192, 204). Results from these studies could not demonstrate significant adverse effects of HC on the developing brain. Thus, HC does not seem to have any large negative impact on brain development in premature infants, although the connection between pathological findings on the MRI of the brain and functional impairment of the cerebellum is not always apparent. It is well known that in some types of progressive ataxia syndromes the MRI of the brain is essentially normal (205, 206), although patients have signs of cerebellar malfunction like ataxia, dysdiadochokinesia and dysarthria. Dyskinetic or ataxic CP has no specific signs on MRI (14). It is likely that the loss of neurons has to be extensive before volume loss can be detected by MRI. Thus, if HC induces a neuronal cell loss in preterm infants, this might be undetectable by current MRI technique. Dex-treated children, on the other hand, showed reduced ABC test performance and a reduction of cerebral gray matter (191). Our chick model and other models suggest dose-dependent effects of HC on cerebellar neuronal death and proliferation (paper 2, 54, 53). Since HC has shown little effect on prevention of BPD in premature infants and at the same time induces dose-dependent neurotoxic effects in animal models, HC may not be the drug of choice for the treatment of BPD.
6 Future perspectives

The chicken embryo cerebellum including cerebellar neuron culture is a useful model for drug-induced CNS effects. Rapid development, independence of host organism, easy and cheap manipulation and also the completed sequencing of the whole chicken genome suggest that this model system would be well suited for further neurotoxicological studies. It has already been implemented in a joint Norwegian safety pharmacology research project granted funding by the Research Council of Norway (ISP-Pharm, Projectno: 195484). The project is aiming at expanding the knowledge and reliability of the existing model. Different agents will be tested for their neurotoxic potency in the model in vivo and in vitro.

Which other important research questions should be addressed next?

Premature birth is often associated with chorioamnionitis and preterm infants have a variable degree of RDS and require - especially with extreme premature gestational age - artificial ventilation leading, among other factors, to a risk for periodical hypooxygenation. Ahlbom et al. (57) have shown that GC treatment renders cells more vulnerable to oxidative stress even after considerably long time after exposure in utero. It would be therefore of interest to test the effect of GCs together with other conditions such as hypoxia and inflammation.

Effects of MK801, a selective NMDA antagonist on GC action has been described in paper 4. It would be interesting to show a possible cross talk between the GR and the NMDA receptor by direct protein technique and to further characterize this interaction. This also applies for the crosstalk between GR and other members of the nuclear family, such as Nurr-1, the predominant member of the NR4 group in the brain.
7 Conclusions

- The chicken embryo cerebellum represents a practical model organ for the study of toxic effects on different aspect of neuronal development. The chicken embryo model is easy to manipulate and generates low costs.
- The chicken cerebellar neuron culture is a highly enriched primary neuronal culture that can be grown under physiological conditions and can be used for various molecular study techniques such as immunochemistry and transfection.
- GCs induce apoptotic death and differentiation and reduce proliferation in granule neurons of the chicken embryo cerebellum and granule neuron culture. Dex was more potent than HC with regard to effect on proliferation and differentiation, but the two agents showed equal magnitude of effect on apoptosis.
- Apoptotic death by GC treatment seems to be initiated through a non-classical steroid action, possibly involving membrane bound GR and cross talk with the NMDA receptor, whereas the GC effect on proliferation appears to be mediated via a classical steroid action.
- Endogenous ligand activated GR action on target genes can be blocked by the orphan nuclear receptor NGFI-B in chicken cerebellar granule neuron culture.
- Since HC has no documented clinical effect on the development of BPD in relevant meta-analyses, but dose-dependent effects on neuronal apoptosis and proliferation in animal models including immature chicken cerebellum, HC may not be the best option for prevention of BPD in preterm infants.
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Transfection of chicken cerebellar granule neurons used to study glucocorticoid receptor regulation by nuclear receptor 4A (NR4A)
Paper 4
Glucocorticoids dexamethasone and hydrocortisone inhibit proliferation and accelerate maturation of chicken cerebellar granule neurons.
(Manuscript)
Glucocorticoids dexamethasone and hydrocortisone inhibit proliferation and accelerate maturation of chicken cerebellar granule neuron precursors

**Short title:** Glucocorticoids and granule neuron proliferation and differentiation

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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.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AraC</td>
<td>Cytosine arabinoside</td>
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<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<td>CCGN</td>
<td>Chicken cerebellar granule neuron</td>
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<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
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<td>Dex</td>
<td>Dexamethasone</td>
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<td>DIV</td>
<td>Days in vitro</td>
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<td>EGL</td>
<td>External granular layer</td>
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<td>GC</td>
<td>Glucocorticoid</td>
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<td>HC</td>
<td>Hydrocortisone/Cortisol</td>
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<tr>
<td>IGL</td>
<td>Internal granular layer</td>
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<tr>
<td>Map2</td>
<td>Microtubule-associated protein 2</td>
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<tr>
<td>NeuN</td>
<td>Neuronal nuclei protein</td>
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<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
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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 (1, 2, 3). One of the target organs of GCs may be the developing cerebellum, as indicated by studies in several animal models (4, 5, 6).

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 at embryonic day (E)16 increases apoptosis in cerebellar granule neurons, both in vivo and in vitro (6). 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 (4). Additionally, GCs are known to promote cell death in neurons in other parts of the brain (7, 8, 9).

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. This differentiation effect may be caused by a block in the G1 phase of the cell cycle, as the antimetabolite and cell cycle inhibitor cytosine arabinoside (AraC) gave a similar differentiation response.
**Material and methods**

*Chicken embryos*

Eggs (weight 50–60 g) from *Gallus gallus* from different hatches were obtained from Samvirkekylling, Våler, Norway. The eggs were incubated in an incubator (Covatutto 20+20, Novital) at 37 °C for 16 days. Dex and HC were injected via openings of about 1 mm into the amniotic cavity (avoiding blood vessels by light transillumination) in three different dosages 24 hours before the embryos were sacrificed. BrdU (80mg/ kg egg) was injected via the same opening 3 hours before the embryo was anesthetized and decapitated. In a subset of experiments RU38486 (20mg/kg egg) was injected into chicken eggs 3 hours prior to injection of Dex and HC. After fixation for 24 h in 4% paraformaldehyde in phosphate buffered saline, the brain was removed from the skull and fixed for a further 24 h. Coronal sections of the brain were routinely processed and slides stained with hematoxylin eosin.

*Chicken granule neuron cultures*

Chicken cerebellar granule neurons (CCGNs) were isolated as previously described 10. Briefly, cerebellar tissue was first treated with BSA and trypsin (Sigma Chemical Co., St. Louis, MO), and then with DNase (Sigma Chemicals Co.), trypsin-inhibitor, and MgSO4. The cell suspension was placed in MgSO4/CaCl2 solution and centrifuged a third time. Then 1.8 × 10^6 cells/ml were seeded onto poly-L-lysine-coated dishes and incubated in BME containing 10% heat inactivated chicken serum at 37 °C and 5% CO2. After 3 days in vitro (DIV) cells were incubated with Dex or HC directly into the medium. In a subset of experiments AraC (Sigma Aldrich Chemie GmbH, Germany) in a concentration of 10 μM diluted in growth medium was added on DIV3. Cells were fixed in paraformaldehyde 4% for 5 min on DIV4.

*Reagents*

Pure Dex and HC (Sigma Aldrich Chemie GmbH, Germany) were dissolved in ethanol and diluted with 0,9% saline. BrdU (Sigma Aldrich Chemie GmbH, Germany) was diluted in saline. All final solutions (including saline control) contained the same amount of ethanol, giving a final ethanol concentration in the chicken egg of approximately 0,2μl/g egg. This had no significant effect on cerebellar granule cell death and proliferation (results not shown).
Detection of cell proliferation and differentiation in chicken cerebellum by morphology and immunohistochemistry

Epitope demasking on slides was performed in a microwave oven for 24 min in target retrieval solution (DAKO AS, Denmark). Cerebellar microsections were then stained with the primary anti-BrdU antibody 1:1600 (Becton Dickinson, USA) or anti-NeuN 1:250 (Chemicon Int. Inc, USA) overnight and then for 2 h with horseradish-peroxidase labeled second layer IgG antibody, followed by diaminobenzidine (DAB) reaction according to the producer’s manual (EnVision+ System HRP, DAKO AS, Denmark). The appropriate blocking solution for endogenous peroxidase was used in the staining procedure. Three images were taken from the IGL of each chicken embryo cerebellum. Images were then processed by Image J software. The ratio BrdU or NeuN positive (brown cell nuclei) versus all cell nuclei (blue nuclei) was calculated.

Double staining of BrdU and Map2

Epitope demasking on slides was performed in a microwave oven for 24 min in target retrieval solution (DAKO AS, Denmark). Cerebellar microsections were then incubated first with primary anti-BrdU antibody 1:1600 for 1 hour and then for 30 min with horseradish-peroxidase labeled second layer IgG antibody followed by DAB reaction. Slides were then washed with H2O and Tris buffer according to the producer’s manual (EnVision™ G|2 Doublestain Rabbit/Mus, DAKO Denmark AS), and incubated with second primary anti-Map2 antibody (Chemicon Int. Inc., USA) 1:2400 overnight 4ºC with alkaline phosphatase labeled secondary antibody for 30 min followed by the Permanent Red chromogen reaction.

Detection of Map2 by Western blot in cerebella

GC treated chicken were sacrificed and the cerebellum was immediately immersed in ice-cold TE buffer (Tris 10mM, EDTA 1mM) with proteinase inhibitors (leupeptin 1μg/ml, pepstatin 1μg/ml, PMSF 0.3mM) and homogenized with 10-12 strokes with the pestle of a glass homogenizer and stored in -70º C. Equal amounts of cell lysates from cerebellum were mixed and boiled with 2 x Laemmli buffer reduced with 2-mercaptoethanol and subjected to electrophoresis on a 10% polyacrylamide gel. Proteins were transferred onto pure nitrocellulose membrane. After protein transfer, the membranes were incubated in TBS-T (100 mM Tris-HCl, 100 mM NaCl, and 0.1% Tween 20) with 5% nonfat milk and primary Map2 (diluted 1:200) followed by a horseradish peroxidase-linked rabbit anti-mouse
antibody (diluted 1:5000). Immunoreactive bands were visualized with Super Signal West Femto Maximum Sensivity Substrate detection kit (Thermo Scientific, USA).

Detection of cell proliferation and differentiation in chicken cerebellar cultures by morphology and immunofluorescence

Fixed cell cultures were incubated with blocking serum for 1 hour and then incubated with primary anti-PCNA antibody (DAKO Denmark AS) 1:200 or anti-NeuN 1:200 overnight. After washing with PBS cell cultures were incubated with secondary Cy3 antibody 1:200 for 1 hour. After washing with PBS nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Photomicrographs were randomly taken with a Nikon fluorescence microscope TE 2000 at 400× magnification from two different places per antigen in two culture dishes for each separate experiment. Images were taken with UV filter (excitation 360–370 nanometers) for the visualization of DAPI, with green filter (excitation 510–560 nanometers) for the visualization of PCNA or NeuN, and then as phase contrast. The percentage of PCNA or NeuN positive cells relative to all DAPI-positive cells was calculated using the NIS Elements software version BR 3.0 (Nikon). Only positive labeled cells, which had granule cell shape in phase contrast, were used for percentage calculation.

Statistical analysis

Group differences were tested either using standard analysis of variance (ANOVA) or Kruskal–Wallis tests depending on whether or not the data passed evaluations for Gaussian distributions. To account for multiple comparisons, we used Dunnett test when comparing treatments to a reference group. All tests are two sided, and results with p<0.05 were considered significant. All estimates are presented with standard error of means when appropriate.
Results

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

We investigated cell proliferation in the cerebellar IGL using BrdU labeling. Chickens at E16 were treated with different dosages of Dex and HC (0.01mg, 0.1mg, 1mg or 5mg/kg egg) for 24 h, and BrdU was added 3 h before sacrifice. The 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, double staining with the neuron marker Map2 showed that most BrdU incorporating cells also expressed Map2 (Fig 1A). Both Dex and HC reduced percentage of proliferating cells relative to all cells in the IGL in a dose-dependent manner (Fig 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 ~ 100-fold difference in potency. The maximum level of reduction of proliferation was the same for HC and Dex, approximately 40%.

*GR antagonist RU 38486 blocks the effect of Dex and HC on proliferation*

To investigate if the effect of Dex and HC was mediated by GR, eggs were pretreated with GR antagonist RU 38486 (20mg/kg egg) for 3 h before treatment with Dex and HC (5mg/kg egg). As shown in Fig 2, inhibition of cell proliferation was blocked.

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

Reduced proliferation is often 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 (11, 12), whereas NeuN is a postmitotic neuronal marker that localizes to the nuclear matrix (13, 14). NeuN expression was investigated 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). Map2 cannot be 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). HC treatment increased mean Map2 expression, but the result was not statistically significant.

*Effect of Dex and HC on proliferation and differentiation is mimicked by AraC in cell culture*
CCGN cultures from chicken cerebellum were treated with Dex and HC. A low dose of Dex (0.1 μM) was chosen because Dex in high dose (10 μM) induces cell death of 50% of cells in culture 6. Low dose Dex 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 (Fig. 4). The reduction detected with HC (10 μM) was less and not statistically significant. The effect of Dex was almost as potent as treatment with the cytostatic agent AraC, used at 10 μM. This concentration of AraC did 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 (Fig. 5 A 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.
Discussion

We have previously investigated the effect of GCs Dex and HC on apoptosis in chicken cerebellar granule neurons, demonstrating induction of apoptosis by both agents in the IGL (6). This paper addresses proliferation and differentiation in the same region and in the same cells of the cerebellum. Although we previously reported non-significant effects of GCs on proliferation in preliminary experiments, more careful investigation revealed a significant, dose-dependent decrease. Thus, injection of a single, pharmacological dose of Dex and HC led to up to 40% inhibition of proliferation, as measured by BrdU incorporation in the IGL at E17. This effect could be blocked by pretreatment of eggs with the GR antagonist RU 38486, and was reproduced in CCGNs in vitro. Dex was about 100-fold more potent than HC, a reflection of their respective pharmacological potency. At E17 the background apoptotic rate is 0.2% versus a proliferation (BrdU positive) rate of 13%. Dex increases the apoptosis rate to ca 0.6% whereas proliferation rate is reduced to 8%. It is, though, difficult to compare these two phenomena quantitively, as apoptotic cells quickly are removed from the tissue whereas BrdU positive cells not undergo removal.

We chose to focus on the IGL in our in vivo experiments. A large fraction of immature granule neurons are located in the EGL, but cells are tightly packed and overlay each other, making cell counting difficult. However, the granule neurons in the purified cultures derive apparently mainly from the EGL (15), and the fact that observed effects in vivo were similar in vitro indicates that EGL neurons respond in the same way as IGL neurons. GC effects on EGL granule neurons have previously been investigated by Bohn et al. (16) in 1-week-old rats, reporting reduced proliferation after treatment with HC. Recently, Heine et al. demonstrated a significant inhibition of proliferation in the EGL after chronic exposure of postnatal mice to Dex and HC (5). They also investigated the relationship of GC-induced growth inhibition with apoptosis, concluding that the contribution of apoptosis was much smaller than proliferation to overall cerebellar growth. In contrast, Noguchi et al. (4) reported a marked increase in apoptosis in the EGL of mice from postnatal day 4 – 10 after a single injection of Dex. We note that in the chicken embryos E16-17 are the most active in terms of spontaneous granule neuron division and apoptosis in the IGL. In our experiments we found significant GC effects on both proliferation and apoptosis occurring at this time point. This period also corresponds to the end of second/beginning of third trimester in human pregnancy, the phase where GCs are given in treatment of premature infants, when comparing the histological picture of the cerebellar layers (17).
Dex and HC also stimulated differentiation, measured as relative increase of Map2 protein expression in cerebellar lysates, and increased fraction of NeuN positive granule neurons. Again, Dex was more potent than HC. Total cerebellar lysates include other cell types than granule neurons, but the latter is dominant in number and it is reasonable to conclude that Map2 increase in granule neurons is significant. Increased fraction of NeuN positive cells was demonstrated in vivo and in vitro and was relative to total number of live neurons. Concomitant apoptosis in vivo conceivable enriches the NeuN positive fraction of cells, and the measured fraction in vivo may therefore be overestimated in our study. However, in vitro, cell death is low and still an increase of the NeuN positive fraction from 40 to 70% was observed. In previous studies the effect of GCs on differentiation markers varies depending on cell type and developmental stage. Similar to our study, Heine and Rowitch (5) recently demonstrated Dex and HC mediated increase in expression of neuronal differentiation marker Zic1 in cerebellar granule neurons of rat pups treated from postnatal day 5 to 7, although Zic1 protein has been demonstrated also in precursor cells and not only differentiated neurons (18, 19). The effect on differentiation may be region dependent as other studies show a reduced expression of Map2 in the hippocampus of young and adult rats when treated with GCs (20, 21). In conclusion, immature neurons appear to respond to GCs by differentiating. Of note, this is paralleled in the immature lung, where GCs are powerful inducers of maturation through surfactant production and several other cellular differentiation effects (22).

The intracellular mechanisms of GC action are remarkably complex, involving both GR mediated transcription, directly through binding to GRE or indirectly through binding to diverse transcription factors, as well as non-transcription dependent events. Many studies have reported a connection between inhibition of proliferation and altered expression of myc, cyclin D, p21 and p27 proteins, leading to a block in the G1 phase of the cell cycle (23, 24, 25). A coupling of myc and cyclin D to exit from G1 has also been shown in immature cortical granule neurons in rat (5). Regarding differentiation, present knowledge is less clear. Several non-neuronal differentiation markers have GRE containing promoters, and interaction with other signaling pathways such as Wnt-betacatenin has recently been demonstrated (26, 27). One important question is how to explain mechanistically the apparent link between GC mediated proliferation and differentiation observed in our study and others. An interesting finding was that the cytostatic agent AraC mimicked the effect of Dex and HC in cell culture on proliferation and differentiation, measured as fraction of cells expressing PCNA and NeuN, respectively. Like GCs, AraC causes a block primarily in the G1
phase of the cell cycle. We therefore hypothesize that the differentiation effect of GC in immature CCGN may be linked to G1 arrest per se, and may not involve specific GC induced genes. Supporting this, there are to date no known GRE in the Map2 and NeuN genes. A considerable body of literature addresses the impact of stress on neuronal development especially in the hippocampus (29, 30) where GCs have been identified as mediators of reduced neuronal proliferation in the dentate gyrus. The present study shows that GCs have the same effect on immature granule neurons in the cerebellum, in addition to previously reported induction of apoptosis. Both of these processes contribute to a reduced number of neurons, and could explain findings of reduced grey matter volume in preterm infants treated with Dex for prevention of bronchopulmonary dysplasia (31). Accelerated differentiation may also be detrimental to cerebellar function and plasticity because of possible reduced neurogenesis and impaired connection of granule neurons especially with Purkinje cells. The effects of Dex were significantly more potent than HC, suggesting a more harmful clinical effect of the former steroid than the latter, in terms of proliferation and differentiation.

In conclusion, this study has shown that the GCs Dex and HC, given as a single dose, inhibit proliferation and increase differentiation of cerebellar granule neurons both in vivo and in vitro in the chicken embryo model. The effects of Dex and HC were inhibited by GR antagonist, and the magnitude of effects reflected their pharmacological potency, suggesting a GR mediated mechanism of action. The differentiation is most likely secondary to the antiproliferative effect of GCs. Both decreased proliferation and early maturation of neurons may lead to reduced cerebellar volume and structural plasticity in the cerebellum, and potentially to the impaired cerebellar function demonstrated in clinical studies.
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**Figure legends**

**Figure 1.** Dex and HC treatments reduce proliferation in a dose-dependent manner in the internal granular layer in chicken cerebellum.

(A) Section shows the IGL and whole cerebellum (inset) of chicken embryo at E17. The neuron specific marker Map2 (red staining) is most intensely expressed in the Purkinje cell layer, but labels the majority of cells of the internal granular layer. BrdU labeled cells (brown) show a scattered distribution in the IGL, and most positive cells also stain with Map2 (×400 magnification).

Chicken embryos at E16 were treated with Dex (B) or HC (C) for 24 h in the given doses. BrdU positive cells were counted on three photomicrographs (×400 magnification) per chicken cerebellum using Image J software. The mean number of BrdU positive cells per internal granular layer on photomicrograph is shown with SEM (n=8 experiments). There was a significant dose-dependent effect of Dex (* p < 0.05, ** p < 0.01, *** p < 0.001) and HC (*p <0.05) compared to control.

**Figure 2.** GR antagonist RU 38486 blocks effects of Dex and HC on proliferation.

Chicken embryos at E16 were treated with Dex (5mg/kg egg) and HC (5mg/kg egg) for 24 h. Some embryos were pretreated (3 hours) with RU 38486 (20 mg/kg egg). Cells incorporating BrdU were counted on three photomicrographs (×400 magnification) per chicken cerebellum using Image J software. The mean number of BrdU positive cells per internal granular layer photomicrograph is shown with SEM (n=3 experiments).

**Figure 3.** Dex or HC treatment induces differentiation markers NeuN and Map2. Chicken embryos at E16 were treated with Dex or HC, both 5mg/kg egg, for 24 h. (A) NeuN positive cells (brown nuclei) were counted on three photomicrographs (×400 magnification) per
chicken cerebellum using Image J software. Representative photomicrographs for each treatment are shown in the upper section. The mean number of NeuN positive cells per internal granular layer on photomicrograph is shown with SEM (n=4 experiments). * p < 0.05 compared to control.

(B) Western blots of whole cell lysates from chicken cerebellum subjected to the indicated treatments. Blots were labeled with antibody against the differentiation marker Map2 and control marker tubulin. One representative blot is shown at the top, and the mean relative optical densities for 9 experiments with SEM are shown at the bottom. * p < 0.05 compared to control.

Figure 4. Dex or cytosine arabinoside (AraC) treatment reduces cell proliferation in chicken cerebellar neuron culture.

Chicken granule neurons in culture were stained with antibody against PCNA 1:200 and DAPI for visualization of the nucleus. Cultured chicken granule cells were treated with ethanol as control, Dex (0.1 μM), HC (10 μM) or AraC (10 μM). Photo images were taken with a Nikon fluorescence microscope TE 2000 at 400× magnification from two different places in two culture dishes/dose and for each separate experiment. Images were shot in the same location, first with UV filter (excitation 360–370 nanometers) for the visualization of DAPI, with green filter (excitation 510–560 nanometers) for the visualization of PCNA and then with phase contrast. The percentages of PCNA positive cells were determined by counting all positive labeled cells with granule cell shape in phase contrast and dividing them by the total number of DAPI-positive nuclei using Nikon software. Means are presented with SEM (n = 6). * p < 0.05 compared to control.
**Figure 5.** Dex or cytosine arabinoside (AraC) treatment induces cell differentiation in chicken cerebellar neuron culture.

(A) Chicken granule neurons in culture were stained with antibody against NeuN 1:200 and DAPI for visualization of the nucleus. DAPI pictures are not shown. Cultured chicken granule cells were treated with ethanol as control, Dex (0.1 μM), HC (10 μM) or AraC (10 μM). Photo images were taken with a Nikon fluorescence microscope TE 2000 at 400× magnification from two different places in two culture dishes/dose and for each separate experiment. Images were shot in the same location with green filter (excitation 510–560 nanometers) for the visualization of NeuN (Cy3) and with phase contrast.

(B) The percentages of NeuN positive cells were determined by counting all positive labeled cells with granule cell shape in phase contrast and dividing them by the total number of DAPI-positive nuclei using Nikon software. Means are presented with SEM (n = 6). * p < 0.05 compared to control.
Figure 1B.
Figure 1C.
Figure 2.
Figure 3A.
Figure 3B.
Figure 4.
Figure 5A.
Figure 5B.