Perinatal glucocorticoid treatment and perspectives for antioxidant therapy

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Perinatal glucocorticoid treatment and perspectives for antioxidant therapy

Perinatale glucocorticoïd behandeling en perspectieven voor antioxidanten therapie
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 30 januari 2014 des middags te 2.30 uur

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Contents

Chapter 1

General introduction

Preterm delivery occurs in approximately 7-10% of all pregnancies in western countries and is the most important cause of infant morbidity and mortality [1]. Due to improvements in both obstetric and, especially, neonatal care, survival of preterm infants has increased substantially over the last decades. The introduction of ante- and neonatal glucocorticoids (GCs) has been an important factor in increasing survival rates and quality of survival of preterm infants.

Antenatal glucocorticoid treatment

Since the pioneering work of Liggins and Howie [2] in the early seventies, synthetic GCs, like betamethasone and dexamethasone, are used worldwide antenatally to treat fetuses at risk for preterm delivery in order to reduce the risk of life-threatening complications of preterm birth including respiratory distress syndrome, necrotizing enterocolitis and intraventricular haemorrhage. Moreover, by decreasing morbidity, antenatal therapy shortens the duration and thereby reduces the costs of neonatal hospitalization. Since the National Institute of Health consensus statement in 1994, a single course of antenatal GC treatment has been recommended for all pregnant women of gestation between 24 and 34 weeks, at risk of delivering within seven days [3]. Antenatal GC treatment involves two maternal intramuscular (i.m.) injections of 12 mg betamethasone 24 hours apart or four i.m. injections of 6 mg dexamethasone 12 hours apart. After crossing the placenta and entering the fetal circulation GC treatment influences the development of the fetus [4]. To exert their effects, GCs bind to the intracellular GC receptor and/or the mineralocorticoid receptor, expressed in almost every organ. In the lungs, GCs stimulate surfactant production, increase lung compliance and maximal volume, promote structural and functional maturation, and regulate pulmonary fluid absorption [5]. Besides beneficial effects, undesirable effects of GCs on other tissues can be expected. In the brain GC receptors are abundantly expressed in the hippocampus [6], an important brain region involved in cognition, behaviour, many memory formation processes, coordination of autonomic functions, regulation of endocrine systems and feedback regulation of the hypothalamic-pituitary-adrenal (HPA)-axis [7,8]. It is therefore likely that among various brain regions the hippocampus appears to be the most vulnerable to antenatal GC treatment and the area of interest of many studies exploring possible adverse effects on brain development. Coe and others [9] have shown that elevated GC levels, caused by prenatal stress, decrease neurogenesis in the dentate gyrus (DG) of juvenile rhesus monkeys and that GCs may suppress cell proliferation. Moreover, dexamethasone administration in adult rats results in death of pyramidal neurons in the cornu ammonal (CA) subfields and granule cells in the DG of the hippocampus [10,11]. Disturbance in development of the hippocampus may have effects later in life. So far in human, the use of a single course of antenatal GCs has not been associated with any significant maternal or fetal adverse effects on the long term [12]. However, data of human follow up studies are only complete up to 30 years of age.

Subgroup analysis of randomized trials, suggested decreased clinical benefit of antenatal GC treatment if birth was delayed more than 7 days after treatment [12]. Furthermore, laboratory evidence showed that fetal surfactant production could be repetitively induced [13], and sheep studies showed that fetal lung maturation was maximal when GCs were administered consecutive over several weeks [14,15]. Therefore, during the nineties, the use of repeated courses of antenatal GCs among pregnant women with prolonged risk of preterm delivery became common clinical practice in many centers. However, since the effects of glucocorticoids are dose dependent, it is likely that adverse effects are more severe when multiple courses are administered. In animal models, an association was found between repeat or high-dose antenatal GCs and reduced fetal growth, long-term adverse effects on brain development, neuroendocrine function, glucose homeostasis and blood pressure [16]. Weekly antenatal GC courses in human do seem to have adverse effects. Although not consistent, several studies found decreased birth weight and head circumference using repeated courses [17-25]. The relative risk of cerebral palsy in infants exposed to repeated courses of antenatal GCs (relative risk, 5.7; 95% confidence interval, 0.7-46.7; P=0.12) in the study of Wapner and co-workers [26] is a serious concern and warrants further study. Because of insufficient scientific evidence and possible harmful effects, the NIH panel recommended that repeat GC courses, including the so-called "rescue therapy," should not be used routinely, but reserved for women enrolled in clinical trials [27]. Because numerous preterm born children received high doses of antenatal GCs, knowledge of expected adverse effects remains important.

Neonatal glucocorticoid treatment

Survivors of preterm delivery may require prolonged mechanical ventilation and may develop chronic lung disease (CLD). CLD is usually defined as oxygen dependency at 36 weeks post menstrual age or 28 days postnatal age, together with persistent clinical respiratory symptoms and concomitant abnormalities on chest radiographs [28,29]. In the eighties Mammel [30] and Avery [31] reported that neonatal/postnatal dexamethasone treatment of preterm neonates significantly improved lung function and allowed earlier weaning from mechanical ventilation in infants at risk for developing CLD.

Although far less research has been conducted on neonatal compared to antenatal GC therapy, a number of studies have highlighted the beneficial effects of neonatal use of GCs, at two key time points after delivery: early (<8 days), and late (>7 days). Given early, GCs facilitate extubation and decrease the risk of CLD and patent ductus arteriosus [32]. If GC therapy was initiated late, treatment was associated with a reduction in neonatal mortality [33]. Late GC treatment also facilitates extubation, it decreases the risk of CLD, and it helps wean infants from mechanical ventilation [33]. Neonatally, dexamethasone has been the GC of choice and commonly administered as a tapering course beginning at 0.5mg/kg. However, the duration of treatment varies from 3 to 42 days [34], and also the timepoint at which treatment is initiated

varies widely. Neonatal GC therapy, with higher dosages and prolonged administration as compared to antenatal therapy, has been shown to have more adverse side effects than the low dose antenatal. The almost routine use of dexamethasone came to an end in 1998, when Yeh *et al.* [35] published the results of a large follow-up study, showing a marked increase in neuromotor dysfunction in male infants treated with dexamethasone, compared to non-steroid treated controls. In the next years, more disturbing publications on long-term negative effects of dexamethasone appeared, reporting adverse effects regarding neurodevelopmental outcome, but also the cardiovascular, endocrine and immune system [36-41], leading to a gradual decrease in neonatal dexamethasone prescription [42]. In 2002, the American Academy of Pediatrics stated that, outside clinical trials, neonatal GC use should be reserved only for "exceptional clinical circumstances". Hydrocortisone, given at a starting dose of 5 mg/kg/day tapering off to 1.25 mg/kg/day over a 22-day period is presented as an alternative to dexamethasone since it has fewer negative long-term side effects [43-46]. However, there have been far less studies on long-term outcome after neonatal use of hydrocortisone in preterm infants than on dexamethasone. Furthermore, no randomized controlled trials comparing dexamethasone and hydrocortisone are published yet. Besides this for many years children have been treated with dexamethasone in high doses and dexamethasone is still used on several neonatal intensive care units worldwide making it very important to get inside in the adverse effects and chances to reduce these adverse effects.

Oxidative stress

Reactive oxygen species (ROS) are products of oxygen metabolism and comprehend oxygen radicals, which possess unpaired electrons, and also non-radical derivatives of oxygen [47]. They are produced by practically all mammalian cells as a byproduct of metabolism and play an important role in normal physiology, serving as signaling molecules in the endothelium, as signaling molecules that induce transcription of several genes and they contribute to the maintenance of vascular tone [48]. However, ROS are also extremely reactive and can cause cellular damage both directly, by attacking proteins, lipids, and polyunsaturated fatty acids, and indirectly, by generating further reactive species and initiating radical chain reactions [49]. Removal of ROS is mediated by antioxidant mechanisms: enzymatic antioxidants that catalyse the disposal of ROS and non-enzymatic antioxidants that scavenge free radicals. A considerable number of studies have suggested that a failure of the balance between pro- and antioxidant mechanisms, or oxidative stress, is involved in the development of several diseases [50-53]. Accumulating evidence suggests that GCs promote oxidative stress, both by enhancing production of ROS and by reducing the levels of endogenous antioxidants [54-57]. Both, GCs and ROS decrease nitric oxide (NO) bioavailability [55,56,58]. Antioxidant treatment has been shown to prevent and partially restore GC-induced vascular dysfunction [56,59] and hypertension [57], suggesting that oxidative stress and subsequent decrease in nitric oxide (NO) bioavailability may be one of the underlying mechanisms mediating adverse effects of GCs. If true, combined treatment with GC and antioxidants may ameliorate the unwanted side-effects of GC therapy in the perinatal period.

Chronic fetal hypoxia

Complications during pregnancy are the most important concern in obstetric medicine, being a major cause of perinatal morbidity and mortality. Reduced oxygenation is one of the most common challenges to the fetus during gestation. It can arise from placental insufficiency [60], high altitude environments [61], maternal respiratory disease [62] or maternal smoking [63]. Reduced oxygenation is thought to cause impairment of cognitive functions as learning disorders [64-67] and is possibly associated with schizophrenia in later life [68]. The mechanism via which adverse intra-uterine conditions programmes fetal development is not well understood, but oxidative stress could be a key link. Hypoxia is a potent stimulus for the generation of ROS [49]. Administration of the xanthine-oxidase inhibitor allopurinol reduces the production of free radicals [69,70]. Furthermore, allopurinol also has a direct free radical (hydroxyl) scavenging effect [71]. Previous studies have shown that allopurinol can limit brain damage by reducing the formation of free radicals after acute asphyxia/hypoxia [69,72,73]. However, not much research has been conducted on the long-term cognitive effects after chronic, prenatal treatment of prolonged but mild hypoxia during pregnancy.

Aim of the thesis

It is established that treatment with GCs in the perinatal period is a life-saving therapy for infants born preterm, but that it also triggers unwanted side-effects. A number of them are known, however there are also some that still need to be explored. Moreover, accumulating evidence suggests that GCs can promote oxidative stress and anti-oxidants can decrease adverse effects. The specific aims of the work described in this thesis are:

Part I

1. To determine effects of a clinically relevant single antenatal GC treatment on hippocampal development in mice.
2. To determine effects of antenatal GC treatment of mothers at risk of preterm delivery on hippocampal histology in human preterm neonates

Part II

3. To determine effects of dexamethasone treatment using a clinically-relevant postnatal dosing regimen on somatic growth, on brain and cardiovascular development in the weanling rat.
4. To determine whether postnatal dexamethasone treatment increases indices of oxidative stress in the brain and decreases NO bioavailability in rats.
5. To determine whether the addition of the antioxidant vitamins C and E or pravastatin ameliorate any detrimental effects of dexamethasone on somatic growth, brain development and cardiovascular development in rats.
6. To determine whether treatment with vitamins C and E or pravastatin alone affects somatic growth, brain or cardiovascular development in rats.

7. To determine effects of postnatal glucocorticoid therapy (dexamethasone and hydrocortisone) on growth patterns in infancy.

Part III

8. To investigate another application of anti-oxidant therapy; to determine whether prenatal hypoxia alters somatic growth anxiety-related behaviour and/or cognitive function in adult offspring.
9. To determine if allopurinol treatment in hypoxic pregnancy ameliorates the adverse effects of hypoxia on behaviour and cognitive function.
10. To determine if allopurinol during normoxic pregnancy affects somatic growth, anxiety related behavior or cognitive function.

Outline of the thesis

The work described in this thesis is divided in three parts. *Part I* highlights effects of antenatal GC treatment on hippocampal histology. In ***Chapter 2*** the correlation between a clinically relevant dose of antenatal GCs on hippocampal development in a mice model is investigated. We investigated the effects on the hippocampus because this is one of the most vulnerable areas of the brain to GC treatment. Because of the alarming findings in chapter 2 we investigated in ***Chapter 3*** the relation between antenatal treatment with GCs and hippocampal development in human neonates.

In *Part II* the focus was shifted to neonatal/postnatal GC treatment. In ***Chapter 4-6****,* in a rat model treatment of postnatal dexamethasone was combined with vitamins C and E or pravastatin, to investigate if adverse effects of GCs on brain and cardiovascular development were reduced. ***Chapter 7*** describes the effects of postnatal GC on growth patterns (body weight, head circumference and length) in a follow up study of preterm born infants treated neonatally with GCs (dexamethasone or hydrocortisone).

In *Part III* another application for antioxidant therapy is investigated, namely in chronic fetal hypoxia. The effects of chronic fetal hypoxia on behaviour and cognitive function in rat adult offspring are investigated, and we determined whether allopurinol had any neuroprotective effects. A summary of the work described in this thesis is provided in ***Chapter 9*** and we also discuss findings here and provide directions for future research.

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PART I

Antenatal Glucocorticoids

Chapter 2

Antenatal glucocorticoid treatment affects hippocampal development in mice

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Abstract

Synthetic glucocorticoids are administered to pregnant women at risk for preterm delivery, to enhance fetal lung maturation. The benefit of this treatment is well established, however caution is necessary because of possible unwanted side effects on development of different organ systems, including the brain. Actions of glucocorticoids are mediated by corticosteroid receptors, which are highly expressed in the hippocampus, a brain structure involved in cognitive functions. Therefore, we analyzed the effects of a single antenatal dexamethasone treatment on the development of the mouse hippocampus. A clinically relevant dose of dexamethasone (0.4 mg/kg) was administered to pregnant mice at embryonic day 15.5 and the hippocampus was analyzed from embryonic day 16 until adulthood. We investigated the effects of dexamethasone treatment on anatomical changes, apoptosis and proliferation in the hippocampus, on hippocampal volume and on total body weight. Our results show that dexamethasone treatment reduced body weight and hippocampal volume transiently during development, but these effects were no longer detected at adulthood. Dexamethasone treatment increased the number of apoptotic cells in the hippocampus until birth, but postnatally no effects of dexamethasone treatment on apoptosis were found. During the phase with increased apoptosis, dexamethasone treatment reduced the number of proliferating cells in the subgranular zone of the dentate gyrus. The number of proliferative cells was increased at postnatal day 5 and 10, but was decreased again at the adult stage. This latter long-term and negative effect of antenatal dexamethasone treatment on the number of proliferative cells in the hippocampus may have important implications for hippocampal network function.

Introduction

Pregnant women at risk for preterm delivery are treated with high doses of synthetic glucocorticoids (GCs), such as dexamethasone (dex) or betamethasone, to enhance fetal lung maturation. Although this treatment is highly effective in reducing mortality and morbidity of the preterm neonate [1], increasing information is available about the adverse side effects of GC treatment. GC treatment directly influences the development of the fetus after crossing the placenta and entering the fetal circulation [2]. The action of GCs is mediated by its interaction with the glucocorticoid receptor and/or the mineralocorticoid receptor, which are abundantly expressed in the hippocampus [3,4]. The hippocampus, an important brain center involved in cognitive functions, can be affected by elevated levels of GCs, by influencing cell death and proliferation. Coe and co-workers [5] have shown that prenatal stress, causing elevated GCs levels, diminishes neurogenesis in the dentate gyrus (DG) of juvenile rhesus monkeys and that GCs may suppress cell proliferation. Moreover, acute administration of dex in rats results in neuronal death of granule cells in the DG, and pyramidal neurons in the cornu ammonal (CA) subfields of the hippocampus [6-8]. In addition, chronically elevated GCs damage hippocampal pyramidal neurons in the CA and inhibit neurogenesis in the adult rat DG [9-12]. Granule cells in the DG are capable of proliferating throughout adulthood by neurogenesis from progenitors located in the subgranular zone of the DG [13,14]. Many studies indicate a relationship between death and birth of neurons and suggest that neurogenesis does occur to maintain neuron numbers, especially after injury [15-17].

Although many studies have focused on GC-induced damage to the hippocampus, little is known about the effects of GCs throughout hippocampal development. To investigate the development of the hippocampus after a single antenatal dex treatment in mice, we used a treatment protocol resembling that used in the human clinical situation and studied apoptosis and cell proliferation and hippocampal volume during prenatal and postnatal life and adulthood.

Materials and Methods

Animals

Pregnant mice C57Bl/6-JIco (Charles River Laboratory, France) were housed individually on day eight of pregnancy. Pregnancy was determined by observation of a vaginal plug. Following timed exposure to the male, the plug date was considered day 0 of gestation. On day 15.5 of pregnancy, the mice were injected intraperitoneally with either dexamethasone (0.4 mg/kg, Dexamethasone Sodium Phosphate) or with equal volumes of sterile saline. Women threatening to deliver preterm, are often administered 6 mg dex four times within 48 hours. With an average weight of around 75 kg, this results in 4 times 0.08 mg/kg dex with a plasma half-life of 3 hours in the human. Plasma half life of dex in mice is unknown; we therefore decided to give one injection of an equivalent dose of dex 0.4 mg/kg. The comparison of the stage of brain development, is a major concern with regard to the interpretation of animal studies looking at the effects of GCs

on neural measures [18]. Estimations of the mouse equivalent age of a term human as respects of neural development have ranged from 5.5 to 19 days of postnatal age [19,20] with a general consensus that a 8- to 11-day-old mouse is equivalent to a term human fetus in terms of brain development. Term mice are therefore best comparable to preterm human fetuses, exactly those who receive prenatal GCs *in utero*. When referring to the administration of dexamethasone in this paper, it should be noted that we have used dexamethasone sodium phosphate like in the clinical situation, which has a larger molecular weight than dexamethasone (516.4 versus 392.5). As a result, a 0.4 mg/kg injection of dexamethasone sodium phosphate is equivalent to a 0.3 mg/kg dose of dexamethasone.

Mice were allowed ad libitum access to food and water. Light/dark cycle (dark phase 1900-0700 h), temperature (21°C) and humidity (60%) were kept constant. Pups were sacrificed by decapitation and studied at seven different time-points; at embryonic day (E) 16, E18, postnatal day (P) 0, P5, P10, P20 and at the age of 6 months. For the adult stage, pups were weaned at P25 and remained group-housed two to four per cage with same-sex littermates until they were sacrificed and studied at 6 months of age (adult). Sixteen randomly chosen pups were sacrificed per time point (dex n=8, sal n=8). Only the heaviest and smallest pup from anyone litter were not included in the experiments. Males and females were equally distributed among the groups. All experimental procedures were approved by the Committee for Animal Experimentation of the University of Utrecht.

Tissue processing

Embryos or brains of mice were dissected and immediately fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C. After fixation, samples were dehydrated and embedded in paraffin. The entire hippocampus was sliced in 7 µm thick coronal sections and mounted on SuperFrost plus slides (Menzel Gläser). All subsequent quantitative analyses were performed with the observer blind to the treatment group.

Nissl-staining

Serial sections obtained from mice at different developmental stages were deparaffinated, rinsed in water, stained for 10 minutes in 0.5% Cresyl Violet and briefly rinsed in an acetate buffer, pH 4. The sections were then differentiated in 96% ethanol for 30 seconds, dehydrated in 100% ethanol, cleared in xylene and mounted with Entellan.

Immunohistochemistry

Serial sections obtained from mice at different developmental stages were deparaffinated, rinsed in water, submitted to microwave treatment (7 min. 650W and 5 min. 350W) in 0.01M Sodium Citrate buffer (pH 6), and incubated in 0.3% H_2O_2 in tris-buffered saline (TBS) for 30 min to reduce endogenous peroxidase activity. Then, sections were washed in TBS, blocked with 4% fetal calf serum in TBS for 30 min and incubated overnight at room temperature with rabbit anti-Ki67 (Chemicon International Inc; 1: 500), or rabbit anti-active caspase-3 (Biovision, USA; 1:100) in TBS. The next day, sections were washed three times with TBS for 5 min, incubated

for 1 h with biotinylated goat anti-rabbit immunoglobulin in TBS (1: 1000), washed three times with TBS for 5 min, incubated for 1 h with avidin-biotin-peroxidase reagents (ABC elite kit, Vector Laboratories, 1: 1000) in TBS and washed with TBS three times for 5 min. The slides were stained with DAB (3,3'-diamino-benzidine), were washed twice with demineralized water for 5 min, dehydrated with ethanol and mounted using Entellan.

Quantification and stereology

Nissl-stained serial sections (7µm) were used for stereological quantification and measurements were performed using an image based analysis system. Neuronal density and volume were calculated in the CA pyramidal cell layer and in the granule cell layer of the dentate gyrus (DG), using the optical dissector method [21]. Object-Image software was used to randomly place a square counting frame over the cell layer on the section. Individual sections were viewed on a video monitor connected to a Zeiss microscope at a final magnification of 40x and counted if they were positioned within the counting frame or intersected by its inclusion edges. The total number of neurons was calculated from the neuronal density and the total volume of the cell layer. Quantification of Ki-67-immunoreactive cells was performed in hippocampal sections (coronal) adjacent to those stained with cresyl violet in the subgranular zone of the DG. The subgranular zone was defined as a two-cell layer thick zone along the inner border of the granule cell layer. Caspase-3 positive cells were counted in the pyramidal cell layer of the CA and in the granule cell layer of the DG.

Statistical analysis

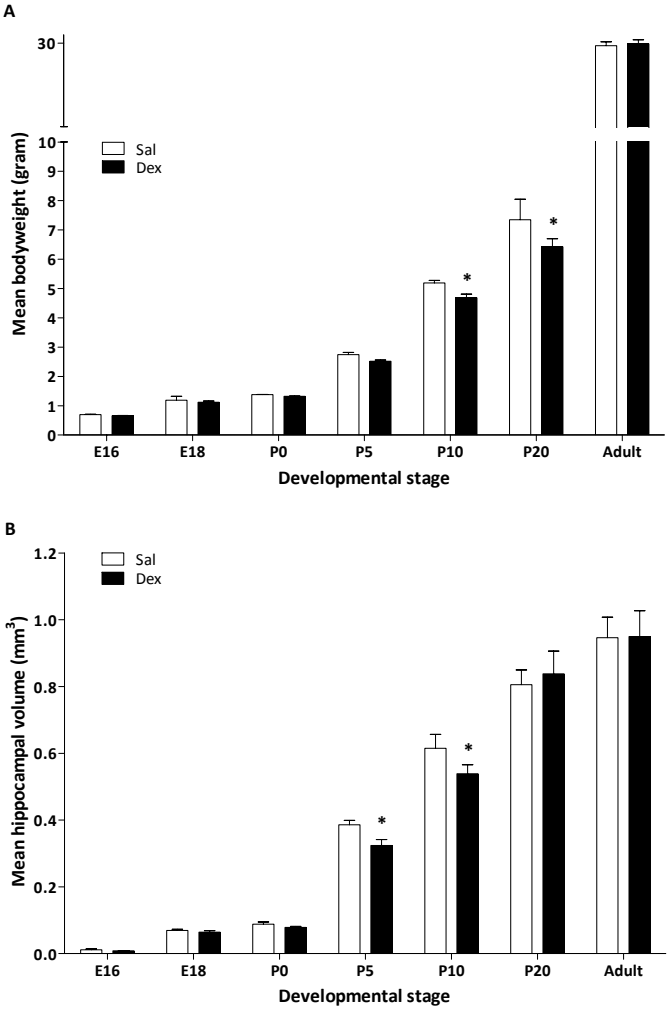
Statistical analysis was performed using Two-Way ANOVA followed by the Bonferroni *post hoc* test. Data are presented as means ± SD. P values <0.05 were accepted as statistically significant.

Results

Body weight and hippocampal volume

Figure 1 shows the effects of a single antenatal dex treatment on fetal, neonatal and adult body weight (Fig. 1A) and hippocampal volume (Fig. 1B). The dex group showed a significantly lower body weight as compared to the saline group at P10 (5.19±0.09g in sal-treated vs.4.68±0.13g in dex treated animals; $P<0.01$) and P20 (sal: 7.35±0.70g vs. dex: 6.42±0.28g; $P<0.0001$). Interaction treatment x time $F(1,98)=7.569$, $P<0.0001$; treatment $F(1,98)=22820$, $P<0.0001$; time $F(1,98)=19.60$, $P<0.0001$. At E16, E18, P0, P5 and adult stage body weights were similar between both groups. Furthermore, after dex treatment a reduction in total hippocampal volume was observed at P5 (sal: 0.386±0.013mm^3 vs. dex: 0.324±0.018mm^3; $P<0.01$) and P10 (sal: 0.615±0.041mm^3 vs. dex: 0.539±0.027mm^3; $P<0.001$). Interaction treatment x time $F(1,98)=4.217$, $P=0.0008$; treatment $F(1,98)=1686$, $P<0.0001$; time $F(1,98)=5.965$, $P=0.0164$. At E16, E18, P0, P20 and adult stage, dex treated animals did not differ in hippocampal volume compared to the saline group.

Figure 1. Effect of antenatal dexamethasone treatment on body weight and hippocampal volume



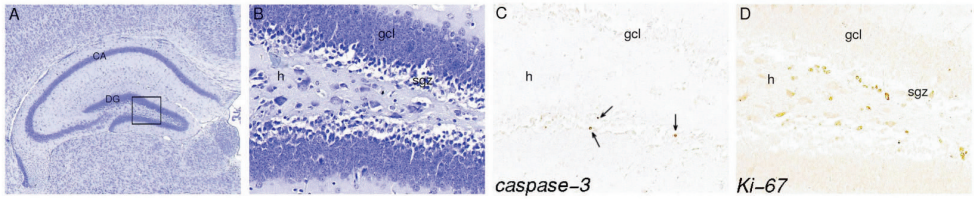
Data are presented as mean ± SD. Dex-treated mice showed a significant reduction in mean body weight at P10 and P20 and reduced hippocampal volume at P5 and P10. E: embryonic day, P: postnatal day, sal: saline-treatment (n=8), dex: dexamethasone-treatment (n=8), * $p<0.05$ (Two-Way ANOVA+Bonferroni).

Volume and cell number

To investigate the effects of dex on the hippocampus, Nissl-stained coronal sections were used (Fig. 2A, B). Examination of the hippocampus did not reveal any disturbances in cellular morphology or anatomical organization after antenatal dex treatment at any of the developmental stages analyzed. The hippocampus was subdivided into CA and DG subregions and volume and total cell numbers were determined in hippocampus sections from E16 till adult stage. Figure 3 shows

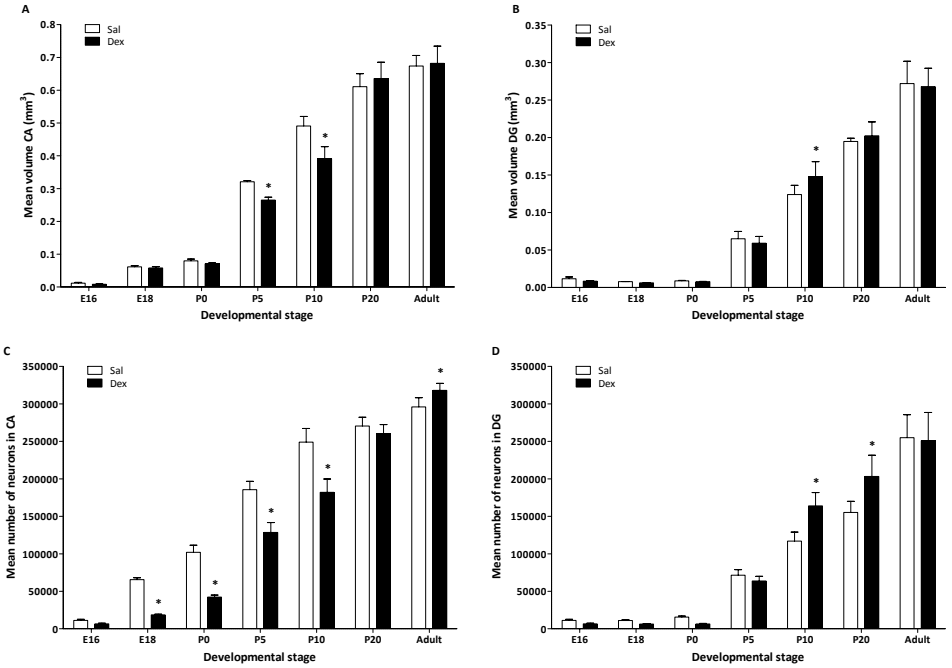
that the volume and total number of neurons in the CA and DG increased during hippocampal development in both the sal and the dex group. In the CA, a significantly lower volume was found after dex treatment, at P5 (sal: 0.321±0.004 mm^3 vs. dex: 0.265±0.009mm^3; P<0.001) and P10 (sal: 0.491±0.029mm^3 vs. dex: 0.391±0.037mm^3; P<0.0001) (Fig 3A). Interaction treatment x time F(1,98)=10.16, P<0.0001; treatment F(1,98)=1673, P<0.0001; time F(1,98)=15.13, P=0.0002. No difference in volume of the CA was detected between the sal and the dex group at E16, E18, P0, P20 and adult stage. However, when we studied the total number of neurons in the CA, we found a significantly lower total number of neurons in the CA at E18 (sal: 65631±2516 vs. dex: 18523±959; P<0.0001) until P10 (P0 sal: 102000±9525 vs. dex: 42300±2622; P5 sal: 185521±11215 vs. dex: 128520±13205; P10 sal: 249000±18288 vs. dex: 182000±17888; all P<0.0001) in the dex treated animals. At adult stage a significantly increased number of neurons was found in these animals (sal: 296000±12297 vs. dex: 318000±9357; P<0.001). Interaction treatment x time F(1,98)=41.16, P<0.0001; treatment F(1,98)=1880, P<0.0001; time F(1,98)=251.9, P<0.0001. The two groups did not differ in total number of neurons in the CA at E16 and P20 (Fig 3C). The DG of the hippocampus had a significantly increased volume after dex treatment compared to the saline group at P10 (sal: 0.124±0.012mm^3 vs. dex: 0.148±0.020mm^3; P<0.01). Interaction treatment x time F(1,98)=2.416, P=0.0321; treatment F(1,98)=967.9, P<0.0001; time NS (not significant, F<1) (Fig 3 B). No difference in volume of the DG was found between the groups, from E16 until P5, at P20 and adult stage. A significantly higher number of neurons in the DG was observed in the dex group at P10 (sal: 117000±11959 vs. dex: 164000±17786; P<0.0001) and P20 (sal: 155255±14717.91 vs. dex: 203194±28303; P<0.0001) (Fig. 3D). At E16 until P5 and at adult stage the groups did not differ in number of neurons in the DG. Interaction treatment x time F(1,98)=9.779, P<0.0001; treatment F(1,98)=540.6, P<0.0001; time F(1,98)=8.564, P=0.0043.

Figure 2. Typical example of a hippocampus of P20, stained with Nissl (A, B),



Panel B, C and D represent a magnification (40x) of the boxed area in panel A (10x). Arrows in panel C show apoptotic cells in gcl. Proliferative cells are detected in the sgz of the DG. h=hilus, gcl=granule cell layer, sgz=subgranular zone.

Figure 3. Effect of antenatal dexamethasone treatment on the volume (A, B) and total number of neurons (C, D) of the hippocampus.



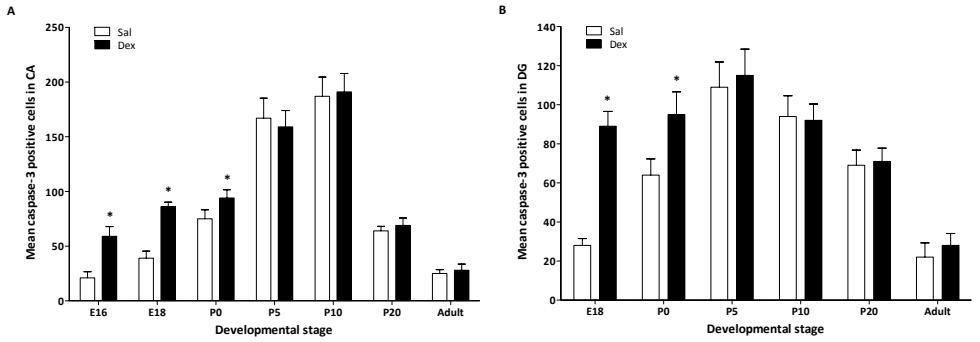
Data are presented as mean ± SD. Panel A and C show a significant decrease in volume of the CA at P5 and P10 and in total number of neurons in the CA area at E18 until P10 and an increase of number of neurons in the CA of the dex-treated group at adulthood. Panel B shows an increase in volume of the DG of the dex-treated group at P10. Panel D shows a significant increase in total number of neurons in the DG of the dex-treated group at P10 and P20. Sal: saline-treatment (n=8), dex: dexamethasone-treatment (n=8). * $p<0.05$ (Two-Way ANOVA+Bonferroni).

Apoptosis

To investigate the effects of antenatal dex treatment on the number of apoptotic cells in the CA and DG, active-caspase-3 positive cells were counted at different stages during hippocampal development (Fig. 2C). Analysis of the number of apoptotic cells started at E18, since in the mouse hippocampus the CA is not distinguishable from the DG at earlier stages of development. In both experimental groups, the total number of apoptotic cells in the CA increased during development until P10, and decreased thereafter (Fig. 4A). The number of apoptotic cells in the DG also increased during development, but already after P5 the number of apoptotic cells decreased (Fig. 4B). Significantly more apoptotic cells were detected after dex treatment in both the CA and DG. In the CA at E16 (sal: 21±5.6 vs. dex: 59±8.9; $P<0.0001$), E18 (sal: 39±6.5 vs. dex: 86±4.2; $P<0.0001$) and P0 (sal: 75±8.2 vs. dex: 94±7.6; $P<0.01$) and in the DG at E18 (sal: 28±3.5 vs. dex: 89±7.6; $P<0.0001$) and P0 (sal: 64±8.3 vs. dex: 95±11.6; $P<0.0001$). No differences in the number of apoptotic cells were detected postnatally (P5, P10, P20 and adult

stage) in either the CA or DG area of the hippocampus between the experimental groups (Fig. 4). Interaction treatment x time active caspase-3 in the CA F(1,98)=14.86, P<0.0001; treatment F(1,98)=555.4, P<0.0001; time F(1,98)=60.35, P<0.0001. Interaction treatment x time active caspase-3 in the DG: F(1,84)=28.34, P<0.0001; treatment F(1,84)=171.8, P<00001; time F(1,84)=86.30, P<0.0001.

Figure 4. Effect of antenatal dexamethasone treatment on the total number of apoptotic cells in the pyramidal cell layer in the CA (A) and in the granule cell layer of the DG (B).

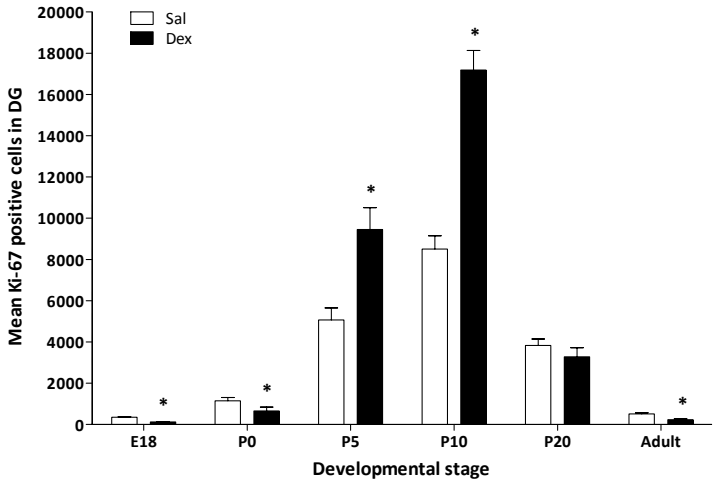


Data are presented as mean ± SD. Apoptosis is significantly increased in the dex-treated group in both the CA area (E16, E18 and P0) and the DG (E18 and P0). Sal: saline-treatment (n=8), dex: dexamethasone-treatment (n=8), * p<0.05 (Two-Way ANOVA+Bonferroni).

Proliferation

The effects of dex on proliferation were visualized using an antibody against Ki-67 (Fig. 2D), a nuclear antigen that is expressed during all stages of the cell cycle except G0 [22,23]. During hippocampal development and in the adult hippocampus, significant numbers of proliferating cells were detected mainly in the subgranular zone (SGZ) along both blades of the dentate gyrus, occasionally in the hilus and only rarely in the granule cell layer (Fig. 2D). Analysis of the number of proliferating cells in the SGZ started at E18, because in the mouse hippocampus the CA is not distinguishable from the DG at earlier stages of development. Immunohistochemical analysis of Ki-67 showed that antenatal dex treatment was associated with a lower number of proliferating cells in the SGZ of the DG compared to the saline-treated group at E18 (sal: 349±29 vs. dex: 125±18; P<0.0001) and P0 (sal: 1148±156 vs. dex: 659±184; P<0.0001) (Fig.5). However, at P5 and P10, the number of proliferating cells was higher in the dex than in the saline group (P5 sal: 5064±584 vs. dex: 9453±1058; P10 sal: 8502±651 vs. dex: 17182±954; both P<0.0001). At P20 no difference was observed while at adult stage the number of proliferating cells was again significantly lower after antenatal dex treatment (sal: 510±60 vs. dex: 230±46; P<0.0001). Proliferation was analyzed as percentage of control (100%). Interaction treatment x time F(1,84)=46.42, P<0.0001; treatment F(1,84)=46.2, P<0.0001; time NS (F<1).

Figure 5. Effect of antenatal dexamethasone treatment on the total number of proliferative cells in the subgranular zone of the DG.



Data are presented as mean ± SD. At E18 and P0, the number of proliferative cells is decreased in the dex-treated group, while an increase is observed at P5 and P10. At the adult stage the number of proliferative cells are decreased in the dex-treated group. Sal: saline-treatment (n=8), dex: dexamethasone-treatment (n=8), * $p<0.05$ (Two-Way ANOVA+Bonferroni).

Discussion

In the present study, we have focused on the effects of a single antenatal dex treatment on the development of the mouse hippocampus. By giving 0.4 mg/kg dex to pregnant mice we have attempted to replicate the human situation of a single course of antenatal GCs. Shortly after dex treatment apoptosis was increased in both the CA and DG and proliferation was reduced in the SGZ of the DG of the fetal hippocampus. This was followed by enhanced proliferation postnatally, but at adulthood, the number of proliferative cells was lower than in the control group. Body weight, hippocampal volume and the total number of neurons in the CA and DG were reduced by dex administration, but these effects were transient and did not persist in adulthood.

We showed that dex treatment decreased the total number of neurons in the CA at E18 until P10 and the volume of the CA at P5 and P10. This decrease is probably caused by increased apoptosis as shown by the large number of apoptotic cells detected directly after the treatment, at E16, E18 and P0. This finding in the CA area of the hippocampus is in agreement with the findings by Haynes et al. [7], who reported dramatic neuronal cell death in the CA area of rats, with the CA1 and CA3 subfields being particularly vulnerable for acute dex treatment. However, they administered a high dose of dex directly in the rat, while we administered a lower dose prenatally to the mother (20 mg/kg and 0.4 mg/kg, respectively). In an observational study in newborn infants who died within 4 days after delivery, we also found a decreased neuronal density in the

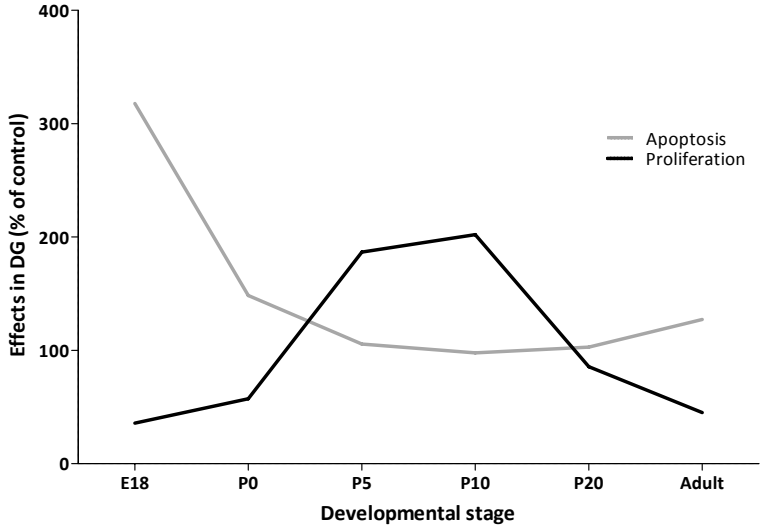
CA area of the hippocampus of neonates treated antenatally with GCs [24]. Chronic stress and long-term administration of GCs have also been found to be associated with loss of hippocampal cells in the CA [25-27]. GC-mediated apoptosis of hippocampal neurons is thought to result from increased expression of proapoptotic genes Bad, Puma and Bnip3 [28-30]. In both experimental groups, the total number of apoptotic cells in the CA and DG increased in the perinatal period (Fig. 4A,B). This increase in apoptotic cells is most likely the result of a rise in endogenous GC release [17], which plays a major role in the preparation for the transition from intrauterine to extrauterine life [31].

In adulthood we found an increase in the number neurons in the CA, with, however, no increase in volume of the CA. This long term increase in the number of CA neurons has never been described before and it will be interesting to investigate what effect this has on hippocampal function.

In the DG, we found an increase in apoptosis and a decrease in proliferation shortly after dex administration at E18 and at birth. However, these effects were not accompanied by a significant effect on the total number of neurons in the DG and on volume of the DG. The effects of dex on apoptosis in the DG are consistent with the findings of Hassan et al. [6], who described GC-induced cell loss in the DG of adult rats after a single dex administration (60 µg/kg). Surprisingly, in the present study, a single antenatal dex administration had similar effects on apoptosis, despite exposure of the fetus to a much lower dose of dex. The inhibiting effect of GCs on neurogenesis in SGZ of the hippocampus is consistent with the findings of others, who described inhibition of granule cell precursor proliferation within 3 hours of GC injection [32-35]. It is thought that GCs decrease proliferation through an N-methyl-D-aspartate (NMDA)-receptor-mediated mechanism [32,36]. The increase in number of neurons in the DG measured at P10 and P20 and the volume at P10 is likely to be the consequence of the large increase (almost doubled) in the number of proliferating cells found at P5 and P10. The large increase in proliferating cells at P5 appears not to be consistent with the measurement of the total number of neurons in the DG at P5, at which time no difference was found after dex administration. However, this apparent 'delay' could be due to the large number of apoptotic cells after dex treatment, which have to be replenished before an increase in total number of neurons can be observed. The increase in proliferation is most likely due to mechanisms involved in neuronal replenishment after apoptosis. The effects of dex on proliferation and apoptosis in the DG during hippocampal development are summarized in figure 6. Many studies have shown a relationship between proliferation and neuron death after ischemia, seizures, brain trauma, and epilepsy [15,16,18,37,38]. Since apoptosis is often associated with increased neurogenesis, it has been proposed that neuronal progenitors may respond to signals from dying cells by re-entering the cell-cycle. One way in which the processes of apoptosis and neurogenesis could be linked is through the regulation of endogenous neurogenic factors [39]. TGF-ß1 is thought to play in important role, since TGF-ß receptors are expressed on proliferating cells in the dentate gyrus, and TGF- ß1 has been shown to be increased under conditions of cell damage. Upregulation of TGF-ß1 after apoptosis may stimulate neuronal progenitors to divide [39]. At P20 and in adulthood, the two groups did not differ in number of proliferative cells, probably because we measured at the end-stage of the

repair mechanism after apoptosis (fig. 6). Finally, at adult stage, when mice are 6 months old, we found a reduced number of proliferating cells in the dex-treated group, while the total number of neurons in the DG was unchanged. It is known that basal neurogenesis declines with advancing age [40], which was also shown in the saline-treated group during hippocampal development. During the life-span the total available number of proliferating cells is more rapidly utilized after apoptosis caused by dex treatment. This long-term effect of dex administration can be due to a restricted amount of proliferation of progenitor cells. Mirescu et al. [41] have shown that the effects of postnatal stress decrease proliferation in adults. Our results indicate that GCs in early life can permanently affect neurogenesis.

Figure 6. Summary of the effects of antenatal dex treatment on proliferation and apoptosis in the DG during hippocampal development.



The results of the dex treated groups are presented as percentage of the control (100%).

Dex treatment induced growth restriction, as measured by body weight, was present at P5 and P10. The decrease in body weight is most likely due to the effects of dex on tissue accretion and catabolism [42-44] and to decreased circulating levels of IGF-1 [45]. The effects of dex on birth weight are consistent with one study in human infants born preterm following a single course of antenatal GCs [46]. Nevertheless birth weights from treatment and placebo groups in several trials are similar and provide reasonable evidence that a single course of GCs in humans does not affect fetal growth [47]. Following repeated antenatal GCs courses a weight restriction at birth has been consistently found [48-51]. As the dex-treated animals themselves were smaller, a decrease in hippocampal volume might be expected, except that the peripheral vasoconstrictor effects of dexamethasone in early life are well known. For instance, two separate studies have reported that treatment of fetal sheep in late gestation with GCs leads to an increase in femoral

vascular resistance [52,53], leading to asymmetric growth, including a brain sparing effect. In this study we did not measure brain weights, we only measured hippocampal volume. Given the fact that in this study significant effects of dex on body weight were found at different time points (P10 and P20) than the effects of dex on hippocampal volume (at P5 and P10), suggests that hippocampal volume is not only determined by body weight.

Fortunately, the results of antenatal dex treatment on body weight, on the volume of the hippocampus and on apoptosis were transient, suggesting compensation and limited long-term effects. The only long-term effects found, were on the number of neurons in the adult mice CA area and on the number of proliferating cells in the adult hippocampus. Accumulating evidence suggests that adult-born dentate granule cells contribute to learning and memory processes, consistent with computational theories that newborn neurons in the networks are likely to be selected for encoding new information (reviewed in Wang et al. [54]). The impaired neurogenesis that we found after antenatal dex at adult stage suggests an impaired cognitive function. Indeed, previously published data by our group indicates that antenatal dex administration results in impaired spatial learning and memory in adult mice [55]. Follow-up studies after antenatal administration of one course of GCs in human is thus far reassuring, with no adverse effects on the child's physical or mental health and psychomotor development at 1 year, 3 years and 6 years. In one study subtle neurological impairment was present at the age of 6, but physical and physiological development at 12 and 20 years were normal [56-59]. Although the general sequence of brain growth and development is similar among species, caution is necessary when extrapolating data from animal models to the human situation. An important consideration is that the maximum velocity of brain growth in mice occurs after parturition, in contrast to humans, where the maximum velocity of brain growth occurs around the time of parturition. The age the pups were exposed to dex treatment was at E15.5, a time point which is comparable to the human situation in the third trimester, as far as hippocampal development is concerned. The dose of dex we used (0.4 mg/kg) was comparable with doses used in other rodent studies published before and almost similar to the human clinical situation were pregnant women receive 4 times a 6 mg intramuscular injection of dex independent of their body weight. Our data suggest that GCs may also in the human affect neurogenesis during adulthood, potentially resulting in cognitive impairment. Such effects might be more pronounced after multiple antenatal courses, a policy that became widespread 10-20 years ago [60,61]. French et al. [62] found that repeated antenatal courses of corticosteroids (≥ 3 courses) were associated with increased rates of aggressive/destructive, distractible, and hyperkinetic behavior at both 3 and 6 years of age. From randomized controlled trials in preterm newborns it has become clear that neonatal GC treatment, in which steroid doses are usually higher and treatment is continued for a longer period, leads to abnormal neurological development, cognitive function and cerebral palsy at follow-up [63-65]. Further detailed follow-up after antenatal dex treatment in the human is therefore recommended and should focus on hippocampal function, for instance spatial learning.

In conclusion, a clinically relevant dose of antenatal dex resulted in increased apoptosis in both the CA and DG and reduced proliferation in the SGZ of the DG of the fetal hippocampus shortly after dex treatment, followed by enhanced proliferation postnatally. However it also caused permanent deficits in proliferation in the adult hippocampus. The latter observations warrant detailed follow up focused on hippocampal function after antenatal GC treatment.

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Chapter 3

Effects of antenatal glucocorticoid therapy on hippocampal histology of preterm infants

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Abstract

Objective

To investigate if antenatal glucocorticoid treatment has an effect on hippocampal histology of the human preterm newborn.

Patients and methods

Included were consecutive neonates with a gestational age between 24 and 32 weeks, who were born between 1991 to 2009, who had died within 4 days after delivery and underwent brain autopsy. Excluded were neonates with congenital malformations and neonates treated postnatally with glucocorticoids.

The brains were routinely fixed, samples of the hippocampus were stained with haematoxylin and eosin and sections were examined for presence or absence of large and small neurons in regions of the hippocampus. Additional staining with GFAP, neurofilament and vimentin was performed to evaluate gliosis and myelination. The proliferation marker Ki67 was used to evaluate neuronal proliferation. Staining with acid fuchsin-thionin was performed to evaluate ischemic damage.

Results

The hippocampi of ten neonates who had been treated with antenatal glucocorticoids showed a lower density of large neurons ($p=0.01$) and neurons irrespective of size ($p=0.02$) as compared to eleven neonates who had not been treated with glucocorticoids. No difference was found in density of small neurons, in myelination, gliosis, proliferation, apoptosis or ischemic damage.

Conclusion

We found a significantly lower density of neurons in the hippocampus of neonates after antenatal glucocorticoid treatment. Although the pathophysiological and clinical interpretations of these findings are not clear, they are consistent with those from experiments in mice and rhesus monkeys.

Introduction

Administration of glucocorticoids (GCs) to pregnant women at risk of preterm birth has led to a major improvement in the outcome of preterm born neonates. Treatment with antenatal GCs reduces the risk of neonatal death, respiratory distress syndrome, periventricular/intraventricular haemorrhage, necrotising enterocolitis, infectious morbidity, need for respiratory support and neonatal intensive care unit admission [1]. Betamethasone and dexamethasone, the synthetic GCs that are given for this indication, cross the placenta and have a high affinity for the GC receptors. These receptors are found in most organs including the brain [2]. GCs promote cellular differentiation at the expense of proliferation resulting into dose related effects in many different species [3]. Regarding the brain there is a dose related reduction in weight in for instance fetal sheep and rat pups [4-6]. In humans, a reduction of head circumference after multiple antenatal GC courses has been found in various observational studies [7-9] and in one out of four recent large randomised controlled trials in which head circumference was an outcome measurement [10-13]. The effects of GCs on brain cell proliferation are most pronounced in areas undergoing active growth and differentiation at the moment of treatment [14]. In this respect, the hippocampal structure, which is part of the limbic system, plays a crucial role in cognitive functions such as learning, memory storage, and spatial orientation and is a metabolic active structure [15,16]. Furthermore, receptors for GCs are highly expressed in the hippocampus [2]. It is therefore likely that among various brain regions the hippocampus appears to be the most vulnerable to antenatal GC treatment. Disturbance in development of the hippocampus may have effects later in life. In monkeys, multiple doses of dexamethasone were associated with a dose-dependent decrease in the number of neurons in the hippocampus as well as degeneration of neurons in this region [17,18]. A study in mice using a clinically relevant dose has established a link between prenatal exposure to dexamethasone and reduced hippocampal volume as well as a reduced number of hippocampal neurons shortly after treatment [19]. Follow-up at adulthood of offspring mice who received corticosteroid treatment in utero showed decreased cell proliferation in the dentate gyrus and adverse effects on hippocampal function [20].
As far as we know, there are no data on the effects of antenatal GC therapy on hippocampal histology of the human fetus or neonate yet. It was our aim to investigate if antenatal GC treatment affects the histological structure of the hippocampus of the fetus and the neonate.

Methods

Ethics Statement

The study protocol was approved by The Ethics Committee of the University Medical Center, Utrecht. They concluded that the Medical Research Involving Human Subjects Act did not apply for this study and that the parents of the research subjects didn't need to be asked for consent. Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our hospital.

Population

From a database containing data on all children born in the University Medical Center, Utrecht, The Netherlands, we identified consecutive neonates with gestational age between 24 and 32 weeks, born between 1991 to 2009, who died during or within 4 days after delivery and underwent a brain autopsy after oral informed consent of the parents. Neonates with congenital malformations or massive brain destruction, such as massive cerebral haemorrhage or encephalitis and/or treated with postnatal GCs were excluded. We reviewed in retrospect the medical files of the included neonates and their mothers. We recorded the reason for admission, use of antenatal GCs, interval between the first gift of GCs and birth, use of postnatal GCs and other drugs, gestational age at delivery, route of delivery, birth weight, sex, parity of the mother, neonatal morbidity, underlying cause and mechanism of perinatal mortality and the interval between birth and death. A complete antenatal GC course consisted of two intramuscular doses of 12 mg betamethasone with a 24-hrs interval. Birth weight percentiles were calculated using birth weight z-scores. Growth charts corrected for gestational age, sex and parity according to the Dutch Perinatal Registry were used to calculate the birth weight z-scores (http://www.perinatreg.nl) [21]. Weight for GA at the 50th percentile was used as the mean of the population and the average standard deviation (SD) calculated by the formula [-1SD + 1SD]/2 was used. Subsequently, the z-score was converted into an exact percentile for each studied subject with a z-score to percentile web calculator (http://www.measuringusability.com). Underlying cause and mechanism of perinatal mortality was based on clinical and pathological findings classified using the Tulip classification [22].

Histological preparation

Brain tissue was fixed in buffered formalin 4%. Four weeks after removal, coronal slices were made and embedded in paraffin after which 3 µm sections were cut and stained with haematoxylin and eosin (H&E). Subsequently the hippocampus was examined for the density of large, small and neurons irrespective of size in all four cornu-ammonal (CA) regions of the hippocampus (CA1, CA2, CA3 and CA4). The density of large and small neurons was scored using semiquantitative analysis comparable with the method used by Groenendaal et al. [23]: high density (4 points), moderate density (3 points), low density (2 points) or (nearly) absent (1 point). Density of neurons irrespective of size was scored by: high (4 points), moderate (3 points), low/moderate (2 points) or low (1 point).

In order to study possible effects of antenatal GC treatment on myelination and gliosis, we performed additional staining on 4 µm sections with antibodies reacting against neurofilament (Monosan, 1:800), vimentin (Dako, 1:400) and glial fibrillary acidic protein (GFAP, Dako, 1:800) on the hippocampus of 11 of the 21 neonates (Table S1, numbers 2-5, 7-9,11,14,15,21). The sections were examined for intensity of staining in all four cornu-ammonal (CA) regions of the hippocampus (CA1, CA2, CA3 and CA4) and scored as: high (4), moderate (3), low (2) or (nearly) absent (1).

Proliferative activity of neurons was evaluated using the proliferation marker Ki67 (Dako, 1:400). The Ki67 protein is expressed in all proliferating cells in late G1, S, G2 and M phases of the cell cycle [24]. Sections were examined for the number of Ki67 positive nuclei in the dentate gyrus

and germinal matrix, two areas where active proliferation usually takes place. The number of Ki67 positive immunoreactive cells was scored as: high (4), moderate (3), low (2) or (nearly) absent (1). To visualise neuronal degeneration and hypoxic ischemic damage a combined staining procedure, acid fuchsin-thionin (0.1% fuchsin, Gurr/BDH; 0.25% thionin, Chroma) was used. Neurons showing ischemic cell change with acidophilic (pink) cytoplasm and contracted nuclei were regarded as nonviable. Sections were examined for intensity of staining, markers of acute ischemia as apoptotic figures and cytotoxic edema in the hippocampus and scored for every category as: high (4), moderate (3), low (2) or (nearly) absent (1).

In order to test the hypothesis that small neurons are mature neurons and large neurons immature neurons, we performed an additional staining with antibodies reacting against doublecortin (1:200, Santa Cruz). Doublecortin is a microtubule-associated protein expressed by neuronal precursor cells and immature neurons.

An experienced perinatal pathologist who was blinded for the treatment of GCs and for the other clinical data, performed the histological examination.

We studied the relation between hippocampal histology and the use of antenatal GCs. Furthermore, relations between the density of large, small or neurons irrespective and gestational age at delivery (≤28 weeks *versus* >28 weeks), birth weight (≤1000 grams *versus* >1000 grams), obstetrical complications (small for gestational age (weight<p10), preeclampsia (PE) and/or HELLP *versus* other pathology), mode of delivery (vaginal delivery *versus* caesarean section (CS)), placenta pathology (small placenta (weight <p10) with infarcts *versus* other pathology), signs of inflammation in the placenta (placenta with chorioamnionitis and/or funisitis *versus* no signs of infection in the placenta) and mechanism of death (respiratory insufficiency *versus* other pathology) were also investigated.

Statistical analysis

Clinical characteristics between the groups were compared by the student t-test or Mann-Whitney U test. Histological data were analysed using the Mann-Whitney U test. SigmaStat software (SigmaStat for Windows, Version 2.0) was used for all statistical analyses. P values <0.05 were accepted as statistically significant.

Results

A total of twenty-one neonates were included. Eleven neonates had not received GCs Ten had received a complete course of antenatal GC therapy. In Table S1, the clinical characteristics of the 21 included neonates are shown. Mean gestational age of the neonates not treated with GCs (n=11) was 27.1 weeks (SD 2.4 weeks) and of the neonates treated with antenatal GCs (n=10) 28.2 weeks (SD 2.1 weeks; p=0.28). Median birth weight was 610 grams (range 450-1700g) in the group not treated with GCs, 865 grams (range 475-1985g) in the group treated antenatally. (p=0.13). Median birth weight percentile was 10.1 (range 0.1-85) in the group not treated with GCs, 38.6 (range 0.4-80) in the antenatally treated group (p=0.11). The median interval between

the first dose of antenatal GCs and delivery was 3 days (range 1-16 days); one neonate had received a repeated course of GCs before birth (number 17). With routine H&E staining we examined in all neonates the presence or absence of large and small neurons in the different hippocampal regions (Figure 1A-D). The nuclei of large neurons showed hypochromatic staining and were large. When present, large neurons showed the highest density in the CA1 region and density gradually decreased in the CA2, CA3 and CA4 region. The nuclei of small neurons were small and round and showed a hyperchromatic staining. When present, small neurons had the lowest density in CA1 and the density increased gradually in the CA2, CA3 and CA4 region. We found no relation between the density of large, small or neurons irrespective of size and gestational age at delivery (≤28 weeks *versus* >28 weeks), birth weight (≤1000 grams *versus* >1000 grams), obstetrical complications (small for gestational age, PE and/or HELLP *versus* other pathology), mode of delivery (vaginal delivery *versus* CS), placenta pathology (small placenta with infarcts *versus* other pathology), signs of inflammation in the placenta (placenta with chorioamnionitis and/or funisitis *versus* no signs of infection in the placenta) and mechanism of death (respiratory insufficiency *versus* other pathology) (Table 1). In hippocampi of neonates treated with antenatal GCs, we found significantly more often a low density of large neurons (p=0.01) as compared to hippocampi of neonates not treated with GCs (Table 1 and 2). No difference in small neurons was found (p=0.35; Table 1 and 2). When we consider the density of neurons irrespective of their size, neonates treated with antenatal GCs showed a significant lower density of neurons in the hippocampus than untreated neonates (p=0.02;Fig 1C,D, Table 1 and 2).

The neurofilament and GFAP staining showed the same low intensity in all hippocampal regions. The intensity of the vimentin staining was the highest in the CA4 region in all hippocampi. In one neonate (number 4) the vimentin staining could not be evaluated because of absence of the hippocampal structure in the additional sections. Because we found no differences at all between the first eleven studied cases for the neurofilament, vimentin or GFAP staining, we terminated this investigation.

Figure 1. Example of the hippocampus of a patient not treated with GCs and of a patient treated with antenatal GCs.

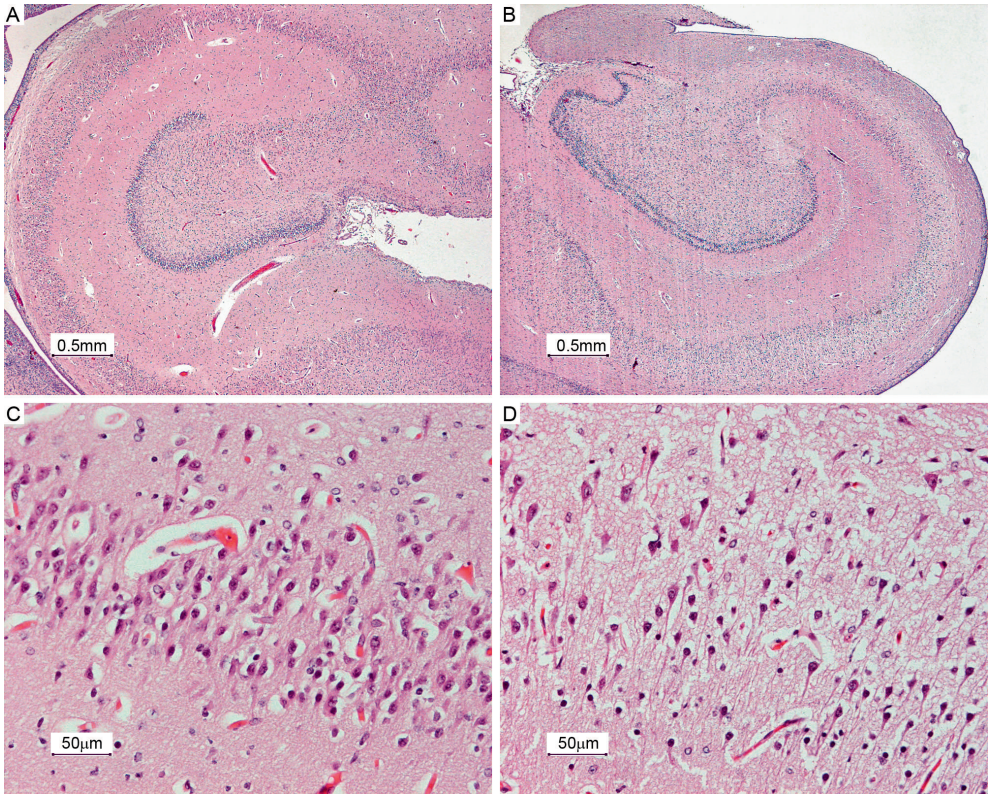


Figure 1A shows an example of the hippocampus of a patient not treated with GCs (number 6) and 1B of a patient treated with antenatal GCs (number 19).
Panel 1C and 1D represent a magnification of transition zone 2 and zone 3 of panel 1A and 1B. Neuronal density is lower in Figure 1D compared to 1C.
Figure 1A and 1B (20x, H&E) and Figure 1C and 1D (200x, H&E).

Table 1. Density scores according to subgroups of patients characteristics.

	Score large neurons	p-value	Score small neurons	p-value	Score neurons irrespective of size	p-value
Antenatal GC treatment						
No AGCs	4.0 [3.0-4.0]	**0.01**	3.0 [1.3-3.0]	0.35	3.0 [3.0-3.8]	**0.02**
AGCs	2.0 [2.0-3.0]		2.0 [2.0-3.0]		2.5 [2.0-3.0]	
GA at delivery						
</= 28 weeks	3.0 [2.0-4.0]	0.29	3.0 [2.0-3.0]	0.13	3.0 [3.0-3.5]	0.07
> 28 weeks	3.0 [2.0-3.0]		2.0 [1.0-3.0]		3.0 [2.0-3.0]	
Birth weight						
</= 1000 grams	3.0 [2.0-4.0]	0.19	3.0 [2.0-3.0]	0.27	3.0 [3.0-3.0]	0.10
> 1000 grams	2.5 [2.0-3.0]		2.0 [1.0-3.0]		2.5 [2.0-3.0]	
Obstetrical complications						
SGA, PE and/or HELLP	3.0 [2.0-4.0]	0.68	3.0 [2.0-3.0]	0.18	3.0 [3.0-3.8]	0.09
Other pathology	3.0 [2.0-3.0]		2.0 [2.0-3.0]		3.0 [2.0-3.0]	
Mode of delivery						
Vaginal delivery	3.0 [2.0-4.0]	0.51	3.0 [2.0-3.0]	0.08	3.0 [2.8-3.0]	0.49
Caesarean section	2.5 [2.0-3.5]		1.5 [1.0-3.0]		3.0 [2.0-3.0]	
Placenta pathology						
Small placenta (>p10) with infarcts	3.0 [2.0-4.0]	0.78	3.0 [1.3-3.8]	0.56	3.0 [2.3-4.0]	0.30
Other pathology	3.0 [2.0-3.0]		2.0 [2.0-3.0]		3.0 [2.0-3.0]	
Signs of inflammation in the placenta						
Chorioamnionitis and/or funisitis	3.0 [3.0-3.3]	0.23	2.5 [2.0-3.0]	0.74	3.0 [3.0-3.0]	1.00
No signs of inflammation	2.0 [2.0-3.8]		2.0 [1.3-3.0]		3.0 [2.0-3.0]	
Mechanism of death						
Respiratory insufficiency	3.0 [2.0-4.0]	0.68	2.0 [2.0-3.3]	0.60	3.0 [2.8-3.3]	0.38
Other pathology	3.0 [2.0-3.5]		2.5 [1.5-3.0]		3.0 [2.0-3.0]	

Numbers represent the median scores, assigned for density of large, small and neurons irrespective of size (see text for details). Between square brackets: p25-p75 range.
Differences are tested with the Mann-Whitney U test.
Abbreviations: AGC = antenatal glucocorticoids, GA = gestational age, SGA = small for gestational age (weight<p10), PE = preeclampsia.

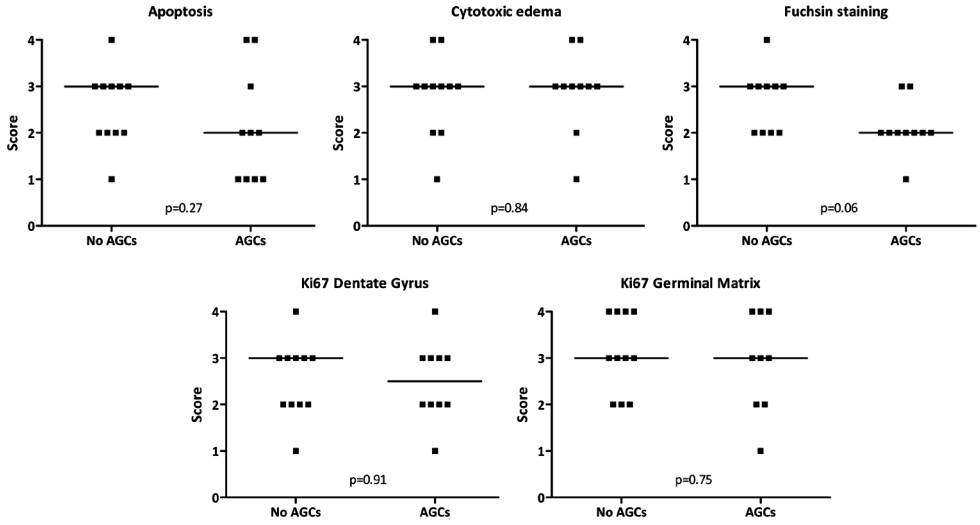
Table 2. Density of large neurons, small neurons, and neurons irrespective of size in the hippocampus of 21 neonates.

	No GCs (n=11)	Antenatal GCs (n=10)	p-value
Large neurons			
High	6	0	
Moderate	3	4	
Low	2	6	
(Nearly) absent	0	0	0.01
Small neurons			
High	2	0	
Moderate	5	3	
Low	1	6	
(Nearly) absent	3	1	0.35
Neurons irrespective of size			
High	3	0	
Moderate	7	5	
Low/moderate	1	4	
Low	0	1	0.02

Numbers represent individual cases.
Differences are tested with the Mann-Whitney U test.

We found no differences in the degree of hypoxic ischemic damage by comparing the presence of apoptotic figures (p=0.27), degree of cytotoxic edema (p=0.84), intensity of fuchsin stained neurons (p=0.06), number of Ki67 positive neurons in the dentate gyrus (p=0.91) and germinal matrix (p=0.75), between the group treated with antenatal GC and the group not treated with GC (Fig 2, Table 3). In one neonate (number 20) the Ki67 staining could not be evaluated in the germinal matrix and in one neonate (number 1) the fuchsin staining could not be evaluated in the hippocampus, both because of absence of these structures in the additional sections. Small and large neurons were both doublecortin immunoreactive. In the brains with most severe damage using acid thionin-fuchsin staining, we detected less doublecortin immunoreactive neurons, supporting the loss of neurons due to hypoxic ischemic damage.

Figure 2. Apoptosis, cytotoxic edema, fuchsin staining and Ki67 positivity in the hippocampus and Ki67 positivity in the germinal matrix of preterm neonates not treated with antenatal GCs and treated with antenatal GCs.



Horizontal axis: the two study groups, vertical axis: staining score (see text for details).
Median values are indicated by horizontal lines. Differences are tested with the Mann Whitney U test.

Table 3. Semiquantitative analysis of the degree of apoptosis, cytotoxic edema fuchsin staining and Ki67 positivity in the hippocampus and of Ki67 positivity in the germinal matrix.

	No GCs (n=11)	Antenatal GCs (n=10)	p-value
Apoptosis	3.0 [2.0-3.0]	2.0 [1.0-3.0]	0.27
Cytotoxic edema	3.0 [2.3-3.0]	3.0 [3.0-3.0]	0.84
Fuchsin staining	3.0 [2.0-3.0][1]	2.0 [2.0-2.0]	0.06
Ki67 staining dentate gyrus	3.0 [2.0-3.0]	2.5 [2.0-3.0]	0.91
Ki67 staining germinal matrix	3.0 [2.3-4.0]	3.0 [2.0-4.0][2]	0.75

Numbers represent the median scores, assigned for high/moderate/low or (nearly) absent and the p25-p75 range between square brackets. Differences are tested with the Mann-Whitney U test.
[1]n=10 cases
[2]n=9 cases

Discussion

To our knowledge, this is the first study evaluating the effects of antenatal GC therapy on the histology of the *human* hippocampus. We found a significantly lower density of neurons in the hippocampus of neonates after antenatal GC treatment (p=0.02). No effect was found on gliosis or myelination after antenatal GC therapy in the eleven cases in which this was studied.

A limitation of this study is the heterogenicity of our patient group. Several pathological conditions during pregnancy, delivery and early neonatal life may have caused hypoxic ischemic damage in the fetus or neonate, a condition in which hippocampal neuronal damage can easily occur. These potentially confounding effects can only be ruled out in a large cohort study with a controlled prospective design. However, for obvious reasons it will not be possible to realise a study with such a design in human neonates. In our study we tried to minimise confounding factors in several ways. First, by including only neonates, who lived for a maximum of four days, limiting influences of early neonatal life. Furthermore, we compared groups for indicators of recent damage, by performing a staining with acid fuchsin-thionin, which highlights neurons with recent hypoxic ischemic damage. No significant differences were found between the two groups. The mode of delivery may affect the density of neurons, but there was no difference in neuronal density between the group born by a vaginal delivery compared to the group delivered by caesarean section.

The results of our study are consistent with earlier investigations using animal models [17-19]. In mice, antenatal dexamethasone treatment significantly decreased the number of neurons in the CA regions of the hippocampus directly after treatment until postnatal day 10 [19]. Also in two of the three studies investigating the effect of antenatal GC treatment on the hippocampus of rhesus monkeys, a decreased number of pyramidal neurons was found, which was ascribed to antenatal GC treatment. Beside this, shrunken neurons with pycnotic nuclei were found in the treated group [17,18]. In the third study these phenomena were found in the treated as well as in non-treated animals and the findings could therefore not be ascribed to the GC treatment [25]. The mechanism behind the decreased neuronal density was not unveiled in this study. Several animal studies have focused on GC-induced hippocampal damage, however very few of them have used clinically relevant doses. Two studies that did use a clinically relevant dose, mention two possible mechanisms; suppression of proliferation *and/or* caspase-3 mediated apoptosis. However these studies are inconclusive. In 2003 Scheepens *et al.* [26] published a study determining the effects of a single course of prenatal betamethasone (2 doses of 170 µgram/kg) within the rat. They demonstrated that betamethasone was antiproliferative to brain cells, including hippocampal cells shortly after treatment for up to 4 days, with some catch-up recovery of the anti-proliferative effects there-after. No changes in caspase-3 mediated apoptosis were seen. In contrast Noorlander *et al.* [19] used a single, somewhat higher, dose of antenatal dexamethasone (1 dose of 400 µgram/kg) to investigate the effects on hippocampal development in mice. They detected, shortly after treatment, beside a decreased proliferative activity in the subgranular zone of the dentate gyrus, the zone where neurogenesis predominantly occurs, increased caspase-3 apoptotic activity throughout the whole hippocampus. The precise mechanism behind the anti-

proliferative effect of GCs on the brain are unknown but possibly involve a down regulation in expression of the trophic growth factors: brain-derived neurotrophic factor (BDNF), IGF-1 and basic fibroblast growth factor (bFGF) [27-29]. Furthermore, administration of any of these three factors has been shown to specifically increase neurogenesis [30,31]. In our study we found no difference in proliferative and apoptotic activity between the antenatal treated group and the control group. An explanation could be that the time interval between corticosteroid exposure and brain analysis may have been too long to detect a possible increase in apoptosis. Another explanation could be that the examined groups were too small to detect a difference.

There was no evidence of a reduction in myelination in our study. However since there is very little myelination in the immature brain between 24 and 32 weeks of gestation, we can not speculate on the possible effects of GCs on myelination given the very low gestational age of the neonates studied. Quantification of myelination at two years of age would be a more appropriate time to examine the effects of antenatal GCs on myelination.

We observed large and small neuronal cells in the hippocampus and both cell types showed its own distribution pattern in the hippocampal regions (large neurons highest density in CA1; small neurons highest density in CA4). By light microscopic examination, both cell types appeared to be neurons of the pyramidal layer of the hippocampus consisting of large and small pyramidal neurons. Our data showed that perinatal GC treatment has more effect on the density of large neurons than on the density of small neurons. We hypothesize that the large neurons might be more immature neurons and the small neurons mature neurons. To test this hypothesis we conducted a doublecortin immunoreactive staining. Both, large and small neurons were doublecortin immunoreactive. Hence, in our study the pathophysiological significance of the density of large and small neurons was not unveiled. Therefore, we also provided the density of all neurons, irrespective of size, which was also lower in the infants treated with GCs (Table 2).

The functional consequences of the effects on hippocampal neuronal density that we observed are unknown. In mice, decreased cell proliferation after exposure to GCs in utero has functional consequences like failure in a number of cognitive and behavioral functions in later life and accelerated aging [20]. However, caution is necessary when extrapolating data from animal models to the human situation.

A study that compared brain weight of neonates who had been delivered between 24 and 34 weeks of gestation showed no significant difference in brain weight between the group that received single or multiple courses of antenatal GCs and a group that did not receive GCs [32]. Thus far, long term outcome after one course of antenatal GCs has been reassuring, and shows only subtle neurological impairment at the age of 6 [33], but normal development in terms of physical, intellectual and emotional development at the age of 12, 20 and 30 years [34-37]. Repeated courses of antenatal GCs, however, have been associated with psychomotor delay and hyperactivity in childhood [38]. Moreover, GC therapy after birth, with higher dosages and prolonged administration as compared to the antenatal therapy, has been shown to increase neuro-developmental impairment [39].

In conclusion, in this unique dataset we found a lower density of neurons in the hippocampus of preterm neonates who were antenatally treated with GCs. We cannot rule out the fact that the observation is caused by other factors as well. Determination of a causal relationship would require a randomized trial. Long-term effects are hard to predict because effects may be transient and the plasticity of the brain at this age may perhaps compensate for a decrease in neurons in the hippocampus. Our observations may cause concern because of the wide use of GC treatment in the perinatal period and indicate that more detailed and specific follow-up studies in humans are required.

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Supporting information

Table S1. Clinical characteristics of 21 included neonates.

	AGCs	GA	Birth weight* (Z-score/ percentile)	Interval first admin. AGCs-birth (days)	Interval birth-death (days)	Obstetrical pathology	Underlying cause of death; subclassification; mechanism[22]	Pathologic examination of the placenta
1	No	24.3	520 (-1.78/p3.8)	-	0	SGA, PPROM, vaginal delivery	Infection; ascending; respiratory insufficiency	Chorioamnionitis, funisitis umbilicalis
2	No	24.6	450 (-2.99/p0.1)	-	0	PE, SGA, Doppler abnormalities, vaginal delivery	Placenta; placental bed pathology; respiratory insufficiency	Small placenta (weight <p10), infarcts (5% of placenta)
3	No	24.7	540 (-1.28/p10)	-	0	PE, placental abruption, vaginal delivery	Placenta; placental bed pathology; respiratory insufficiency	Placental weight p10, placental abruption, infarcts (10% of placenta)
4	No	24.7	780 (0.54/p70)	-	2	Dichorionic twins, intra-uterine infection, vaginal delivery	Infection; ascending; cardiocirculatory insufficiency	Chorioamnionitis
5	No	25.6	525 (-1.54/p6)	-	0	HELLP syndrome, SGA, vaginal delivery	Placenta; placental bed pathology; respiratory insufficiency	Small placenta (weight <p10), infarcts (5% of placenta)
6	No[1]	26.9	665 (-0.72/p23)	-	0	PE, HELLP syndrome,vaginal delivery	Placenta; placental bed pathology; respiratory insufficiency	Small placenta circumvallata (weight <p10), infarcts (<5% of placenta), increased maturation
7	No	28.0	540 (-1.55/p6)	-	4	SGA, Doppler abnormalities, CS	Placenta; placental bed pathology; respiratory insufficiency	Infarcts (10% of placenta), increased maturation
8	No	28.6	610 (-1.40/p8)	-	3	SGA, fetal distress, CS	Placenta; placental bed pathology; cardiocirculatory insufficiency	NA
9	No	29.4	870 (-1.20/p11)	-	2	Fetal distress, CS	Placenta; placental bed pathology; cardiocirculatory insufficiency	Signs of circulation abnormalities, increased maturation
10	No	30.0	1700 (1.04/p85)	-	2	Placental abruption, CS	Placenta; placental bed pathology; cerebral insufficiency	Signs of fetal thrombosis
11	No	31	1210 (-0.56/p29)	-	0	Polyhydramnios, monochorionic twins, vaginal delivery	Placenta; placental pathology-development; cardiocirculatory insufficiency	Monochorionic-diamniotic placenta with some connecting vessels
12	Yes	25.6	475 (-2.65/p0.4)	2	0	Dichorionic twins, SGA, vaginal delivery	Placenta; placental bed pathology; respiratory insufficiency	Diamniotic-dichorionic placenta, small placenta (weight <p10), infarcts (10% of placenta), increased maturation

	AGCs	GA	Birth weight* (Z-score/ percentile)	Interval first admin. AGCs-birth (days)	Interval birth-death (days)	Obstetrical pathology	Underlying cause of death; subclassification; mechanism[22]	Pathologic examination of the placenta
13	Yes	26.1	840 (-0.05/p48)	2	1	Spontaneous preterm delivery, CS, first neonate of quadruplet	Prematurity/immaturity; preterm labour; respiratory insufficiency	Quadruplet placenta, no abnormalities
14	Yes	26.4	550 (-1.33/p9)	3	0	Monochorionic twins, SGA, spontaneous preterm vaginal delivery	Placenta; placental pathology-development; cardiocirculatory insufficiency	Monochorionic-diamniotic placenta, TTTS
15	Yes	26.4	760 (-0.71/p24)	3	0	PPROM, vaginal delivery	Prematurity/immaturity; PPROM; respiratory insufficiency	Chorioamnionitis
16	Yes	27.7	890 (0.10/p54)	4	2	PPROM, suspected intra-uterine infection, vaginal delivery	Prematurity/immaturity; PPROM; respiratory insufficiency	Chorioamnionitis, funisitis umbilicalis
17	Yes[1,2]	28.3	770 (-0.66/p26)	16	1	Dichorionic twins, HELLP syndrome, vaginal delivery	Placenta; placental bed pathology; cardiocirculatory insufficiency	Diamniotic-dichorionic placenta, increased maturation, partial placental abruption
18	Yes	29.1	1300 (0.24/p59)	7	0	PPROM, vaginal delivery	Prematurity/immaturity; PPROM; cardiocirculatory insufficiency	Increased maturation, chorioamnionitis, funisitis umbilicalis
19	Yes	30	1200 (-0.55/p29)	4	3	Fetal distress, CS	Placenta; placental bed pathology; cerebral insufficiency;	Small placenta (weight <p10), infarcts (5% of placenta), partial placental abruption
20	Yes	30.3	1565 (0.71/p76)	4	0	PPROM, suspected intra-uterine infection, CS	Prematurity/immaturity; PPROM; cardiocirculatory insufficiency	Chorioamnionitis
21	Yes	32	1985 (0.83/p80)	3	4	HELLP syndrome, fetal distress, CS	Placenta; placental bed pathology; cardiocirculatory insufficiency	NA

Abbreviations: AGCs = antenatal glucocorticoids, Admin. = administration; CS = caesarean section; GA = gestational age at delivery; SGA = small for gestational age (weight <p10); PE = preeclampsia; PPROM = preterm premature rupture of membranes; TTTS = twin to twin transfusion syndrome; NA = Not available

* Birth weight in grams

[1] Enrolled in HELLP trial (high dose prednisolone versus placebo)

[2] Repeated courses antenatal GCs

PART II

Postnatal glucocorticoids

Chapter 4

Oxidative stress in the developing brain: effects of postnatal glucocorticoid therapy and antioxidants in the rat

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Abstract

In premature infants, glucocorticoids ameliorate chronic lung disease, but have adverse effects on long-term neurological function. Glucocorticoid excess promotes free radical overproduction. We hypothesised that the adverse effects of postnatal glucocorticoid therapy on the developing brain are secondary to oxidative stress and that antioxidant treatment would diminish unwanted effects. Male rat pups received a clinically-relevant tapering course of dexamethasone (DEX; 0.5, 0.3, and 0.1mg.kg^{-1}.day^{-1}), with or without antioxidant vitamins C and E (DEXCE; 200mg.kg^{-1}.day^{-1} and 100mg.kg^{-1}.day^{-1}, respectively), on postnatal days 1-6 (P1-6). Controls received saline or saline with vitamins. At weaning, relative to controls, DEX decreased total brain volume (704.4±34.7mm^3 vs. 564.0±20.0mm^3), the soma volume of neurons in the CA1 (1172.6±30.4μm^3 vs. 1002.4±11.8μm^3) and in the dentate gyrus (525.9±27.2μm^3 vs. 421.5±24.6μm^3) of the hippocampus, and induced oxidative stress in the cortex (protein expression: heat shock protein 70 [Hsp70]: +68%; 4-hydroxynonenal [4-HNE]: +118% and nitrotyrosine [NT]: +20%). Dexamethasone in combination with vitamins resulted in improvements in total brain volume (637.5±43.1mm^3), and soma volume of neurons in the CA1 (1157.5±42.4μm^3) and the dentate gyrus (536.1±27.2μm^3). Hsp70 protein expression was unaltered in the cortex (+9%), however, 4-HNE (+95%) and NT (+24%) protein expression remained upregulated. Treatment of neonates with vitamins alone induced oxidative stress in the cortex (Hsp70: +67%; 4-HNE: +73%; NT: +22%) and in the hippocampus (NT: +35%). Combined glucocorticoid and antioxidant therapy in premature infants may be safer for the developing brain than glucocorticoids alone in the treatment of chronic lung disease. However, antioxidant therapy in healthy offspring is not recommended.

Introduction

Glucocorticoids are widely used to treat or prevent chronic lung disease (CLD) in preterm infants [1-5]. A major contributor to the development of CLD is excessive inflammation and thus glucocorticoids are used to prevent or reduce the severity of this complication due to their anti-inflammatory properties [4,6,7]. Despite established benefits, randomized clinical trials of postnatal steroid therapy have raised concerns regarding an increase in the rates of cerebral palsy and adverse neuromotor and cognitive outcomes [8,9]. Furthermore, experimental studies in neonatal animals have demonstrated adverse effects of potent glucocorticoids, such as dexamethasone on brain growth, cell division, differentiation, myelination, apoptosis, and neurogenesis [10-15]. Clinically, therefore, the weaker glucocorticoid hydrocortisone is the steroid of choice [16]. The use of dexamethasone being particularly reserved for extremely premature (< 32 weeks) and low birth weight infants unlikely to survive without therapy. However, whilst hydrocortisone appears to cause fewer unwanted adverse effects than dexamethasone, it is also less potent in promoting the beneficial effects of glucocorticoids [16-18]. For these reasons, there is increasing clinical and basic science interest in persisting with the clinical use of potent steroids, such as dexamethasone, as long as the adverse side effects can be controlled. However, the mechanism underlying the unwanted side effects remains unknown, preventing the identification of plausible modified therapies to maintain the beneficial effects but prevent adverse side-effects.

Accumulating evidence suggests that glucocorticoids can promote oxidative stress in various tissues including the heart and vasculature [19-23] and kidney [20]. In relation to the brain, glucocorticoids increase the presence of reactive oxygen species (ROS) in both hippocampal and cortical cultures [24], and lower basal activity of cerebral antioxidant enzymes [25]. Furthermore, *in vitro* studies have shown that exposure to glucocorticoids can increase the susceptibility of cerebellar granule cells [26] and hippocampal neurons [27] to oxidative stress-induced cell death. Reactive oxygen species are produced under physiological conditions during metabolic reactions and functioning of the central nervous system [28]. However, excess ROS generation can cause cellular damage directly by attacking proteins, and indirectly, by generating further reactive species and initiating radical chain reactions [29]. Non-enzymatic and enzymatic antioxidants provide defence mechanisms against excess ROS production. The former include the water soluble vitamin C and the fat soluble vitamin E. Vitamin C is the most effective aqueous-phase antioxidant in human blood plasma because of its ability to trap peroxyl radicals, thereby preventing lipid peroxidation [30]. Vitamin E is a peroxyl radical scavenger and one of the most important inhibitors of the free-radical chain reaction of lipid peroxidation, giving it a major role in protecting biological membranes [31]. Antioxidant treatment has been shown to prevent and partially restore glucocorticoid-induced vascular dysfunction [22] and hypertension [23], further supporting the premise that oxidative stress may be the underlying mechanism mediating unwanted side-effects of glucocorticoids.

Whilst ROS have been implicated in the pathogenesis of various neurodegenerative disorders including Parkinson's and Alzheimer's disease [32-34], the role of ROS in the pathogenesis of glucocortocoid-induced brain injury during early development, remains unclear. In this study, we have combined these separate lines of evidence to propose the inter-related hypotheses that the adverse effects of postnatal glucocorticoid therapy on the developing brain are secondary to oxidative stress and that antioxidant treatment would diminish unwanted effects. The hypothesis was tested using a well established model of postnatal glucorticoid therapy in rats [35,36].

Methods

Ethical Approval

The study was approved by the Cambridge University Ethical Review Committee. All procedures were carried out under the UK Animals (Scientific Procedures) 1986 Act and conducted under the authority of Project Licence 80/2232.

Animals and Experimental Design

Twenty-five pregnant Wistar rats (Charles River, UK) with timed gestations were individually housed under standard conditions (23±1°C, light:dark, 12:12 hour) with access to food (Special Diet Services, UK) and water. All dams delivered on day 22 of gestation (assigned postnatal day 0, P0). Litters were then divided into four treatment groups: control (Ctrl, n=6), dexamethasone (DEX, n=6), dexamethasone with vitamins C and E (DEXCE, n=7), and control with vitamins C and E (CtrlCE, n=6).

Within 3–5 hours of birth, pups were sexed and weighed, and litters reduced to 8 pups per dam (four males and four females) in order to standardize postnatal nutrition and maternal care. To account for sex differences, only male pups within each litter received treatment (Ctrl, n=24; DEX, n=24; DEXCE, n=28; and CtrlCE, n=24). Male pups received two intraperitoneal (i.p.) injections daily (10 $\mu L.g^{-1}$ for each) of some or all of the following solutions from Sigma (Sigma-Aldrich, UK): Dexamethasone (Dexamethasone-21-phosphate, disodium salt), vitamin C (L-ascorbic acid), and vitamin E (*dl*-α-tocopherol acetate). Two injections were used due to the different solubilities of vitamin E (dissolved in groundnut oil) and vitamin C and dexamethasone (both dissolved in 0.9% NaCl). Ctrl pups received injections of saline and groundnut oil for the duration of the treatment period, postnatal days 1-6 (P1-6). Dexamethasone pups received a three-day, tapering course of dexamethasone (0.5, 0.3, and $0.1 mg.kg^{-1}.day^{-1}$) plus separate injections of oil on P1-P3 and then only saline and oil from P4-P6. DEXCE pups received the same treatment as DEX pups, except that vitamins C ($200 mg.kg^{-1}.day^{-1}$) and E ($100 mg.kg^{-1}.day^{-1}$) were administered over the entire treatment period in addition to the three-day tapering course of dexamethasone. CtrlCE pups received injections of vitamin C and E from P1-6. The dose and duration of dexamethasone used in this study was derived from and is proportional to the 21-day tapering course of dexamethasone used in human preterm infants to prevent or lessen CLD, starting at $0.5 mg.kg^{-1}$ [35]. Ascorbic acid and α-tocopherol are often combined to promote antioxidant activity; this combination

is essential for preventing pro-oxidant activity of the α-tocopheroxyl radical, which is formed from α-tocopherol during lipid peroxidation [31,37]. Ascorbate regenerates α-tocopherol from α-tocopheroxyl radicals, thus preserving the antioxidant role of α-tocopherol [37,38]. The doses of vitamins C and E used in this study were adopted from studies indicating successful antioxidant effects in adult Wistar rats at these levels [39]. Body weight (BW) from P0 to P7 and every other day thereafter was recorded.

Tissue collection

During the treatment period, 10 male DEX pups, 2 male DEXCE pups and 1 male Ctrl pup died, leaving the following remaining male pups for study (Ctrl, n=23; DEX, n=14; DEXCE, n=26; and CtrlCE, n=24). On P21, approximately half of these surviving male pups from each litter (Ctrl: n=11; DEX: n=7; DEXCE: n=12; CtrlCE: n=12) were deeply anaesthetised (0.2mL total volume, i.p., 100mg.mL^{-1} ketamine, Fort Dodge Animal Health, UK and 20mg.mL^{-1} xylazine, Millpledge Veterinary, UK). To assess the symmetry of growth, BW and crown-rump length (CRL) were determined. Brain tissue was weighed and dissected. The hippocampal formation was separated from the overlying cortex using a dissecting microscope [40], then snap frozen in liquid nitrogen and stored at -80°C for subsequent Western blot analysis. On P22, the remaining male pups (Ctrl: n=12; DEX: n=7; DEXCE: n=14; CtrlCE: n=12) were deeply anaesthetised as described above and perfused intracardially with a NaCl solution (10mM PIPES, 139mM NaCl, 2.7mM KCl, 19.4mM D-glucose, 7.5μM PVP, pH 7.2) followed by 4% paraformaldehyde (PFA). Brains were collected, weighed and stored overnight at 4°C in 4% PFA for subsequent stereological analysis. Dissection for isolation of tissue for freezing and fixing was performed on separate days to accommodate the number of animals to be processed within a 24-hour period.

Histology and stereology

Tissue preparation

The cerebrum was exhaustively sectioned at 50μm using a Leica RM 2235 vibratome (Leica Microsystems, Germany). Tissue was then stored in cyroprotectant at -20˚C until histological and immunohistochemical staining was performed. All subsequent quantitative analyses were performed with the observer blind to the treatment group.

Immunohistochemistry

Sections were washed for 30 minutes in phosphate buffered saline (PBS, Sigma-Aldrich, U.K.), incubated with 30% H_2O_2 for 5 min to block endogenous peroxidase activity and washed in PBS for 30 minutes. Subsequently non-specific binding was blocked with 4% BSA in PBS for 10 min. Sections were then incubated with primary antibody (MBP; 1:400, Millipore, UK; NeuN, 1:400, Millipore, UK) in primary diluent (2% BSA in PBS containing 0.3% Triton; Sigma Aldrich, U.K.) overnight. The following day sections were washed 30 minutes in PBS, incubated for 1 hour with secondary antibody (1:400, Vector Laboratories, USA) in secondary diluent (2% BSA in PBS) then washed for 15 minutes in PBS. Sections were incubated for 1 hour in AB (Vector Laboratories, U.K.) in PBS then washed in PBS for 15 minutes. Staining was visualized by adding metal DAB (Thermo Scientific, UK) in peroxide buffer (Thermo Scientific, UK) for 2 minutes to the sections.

Tissue was then mounted with 0.5% gelatine in PBS on slides.

Volumetric analysis

To assess the volume of the cerebrum and its compartments, systematic random sampling [41] was used to select, without bias, 10 sections per animal for analysis. Selected sections were stained using 1% Cresyl Violet. A point grid was superimposed on the sections and viewed using a x1.25 objective. The volume of both the left and right hemispheres, the cortex, white matter, and the hippocampus, were analysed. Points falling on each compartment were counted and the Cavalieri principle (Gundersen et al, 1998) was applied in order to calculate estimated volumes:

$$V_{(obj)} = t \times \Sigma a = t \times a_{(p)} \times \Sigma P$$

where $V_{(obj)}$ represents the estimated volume of the brain region, t is the total length of the brain (t = no. of sections x section thickness), $a_{(p)}$ is the area associated with each point and ΣP is the sum of points for that formation. All volumetric quantifications were performed with an Olympus BX-50 microscope and CAST grid.

Neuronal number and soma volume quantification

Neuronal number was estimated by using the fractionator method. All available NeuN stained sections containing the cortex and hippocampus (divided into the CA1, CA2/3 and dentate gyrus) were sampled using an Olympus BX-50 microscope and CAST grid (Fig. 1). Step motors on the microscope were used to randomly sample a known fraction of the tissue. This was achieved by calibrating the lengths of the X and Y steps. An unbiased counting frame of known dimensions was superimposed on the tissue image. Nuclei within the counting frame, or those touching the permitted lines of the counting frame, were counted. To calculate total neuronal number, the following formula was applied:

$$\text{est } N = \Sigma \text{ n} \,.\, f_1 .\, f_2$$

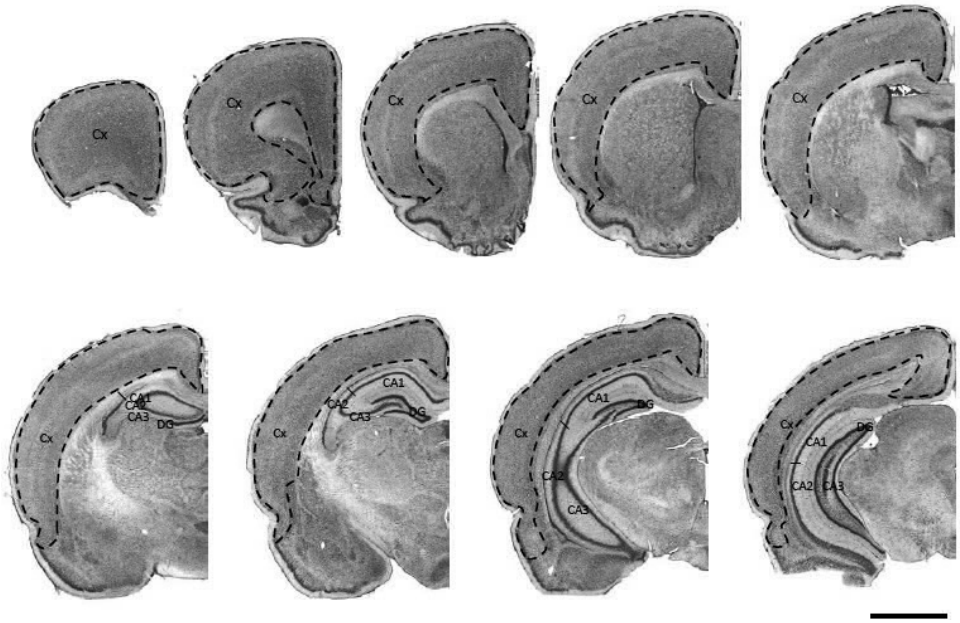
where est N is the estimated total number of nuclei, Σ n is the sum of nuclei counted in the brain sample, f_1 is the reciprocal of the sampling fraction, and f_2 is the areal sampling fraction.

The soma volume of neurons from the cortex and the different regions of the hippocampus were calculated using the vertical dissector tool in the CAST grid programme, which generates two orthogonal lines through the mid-point of the neuron. The boundaries of the neuron were then identified at the intersections of these lines with the cell membrane. Soma volume was calculated using the following formula:

$$Vn = 4\pi/3 * I3n$$

where In refers to the distance from the mid-point of the neuron to the cell membrane of the neuron.

Figure 1. Photomicrograph of coronal brain section.



Cresyl violet-stained sections showing the regions used for determining neuronal number and soma volumes. These parameters were measured in the following fields: cortex (Cx), CA1, CA2/3 and dentate gyrus (DG). Scale bar= 2.5mm.

Myelination

To assess the extent of myelination, the optical density (OD) of MBP-stained fibres was measured in the corpus callosum and cortex using ImageJ (V1.38, National Institutes of Health). On the basis of anatomical landmarks, equivalent sections from all rats were chosen. A calibrated optical density step tablet was used to calibrate the images to optical density. Eight fields within the corpus callosum and cortex were examined over three sections per animal. Optical density was measured as grey levels. Non-specific background ODs were measured at each brain level in a region devoid of MBP-immunostaining and these were subtracted from the corpus callosum and cortex values. The area of MBP-positive fibres in the cortex, relative to cortical size, was also assessed using the same software.

Western blotting

Western blots were performed using 20µg aliquots of protein, resolved on 10-12% SDS-PAGE gels. Proteins were transferred to polyvinylidene fluoride Immobilon-P membranes (Millipore, UK) by electroblotting. Membranes were blocked at room temperature for 1 hour with 5% dry skim milk in tris-buffered-saline containing 1% Tween-20 (TBS-T, Sigma-Aldrich, UK). Purified antibodies to β-actin (1:50,000, Sigma-Aldrich, UK), nitrotyrosine (NT; 1:2,000, Zymed Laboratories, USA), 4-hydroxynonenal (4-HNE; 1:2,000, Calbiochem, Germany), or heat shock protein 70 (Hsp70;

1:20,000, Stressgen, UK) in 5% milk in TBS-T were added, and incubated at 4°C overnight. Membranes were washed in TBS-T, incubated for 1 hour in a secondary antibody conjugated to horseradish peroxidase (donkey anti-rabbit IgG or sheep anti-mouse IgG; 1:10,000, GE Healthcare, UK) and washed in TBS-T. Proteins were visualised using enhanced chemiluminescence (ECL, Amersham, UK), exposed to X-ray film and films were developed (Fuji FPM100A Processor). Bands densities were quantified and expressed relative to β-actin (ImageJ software, NIH).

Statistical Analysis

Data are presented as mean ± SEM unless otherwise indicated. Data were analysed by One-Way ANOVA followed by the Student-Newman-Keuls or Tukey *post hoc* test. SigmaStat software (SigmaStat for Windows, Version 2.0) was used for all statistical analyses. P values < 0.05 were accepted as statistically significant.

Results

Body and brain weights

Compared to control offspring, DEX, with or without vitamins, reduced body weight at weaning (Fig. 2A; $P<0.05$) Absolute brain weight was not altered between the groups (Fig. 2B; $P>0.05$), however, the brain to body weight ratio was significantly increased in DEX offspring, but not DEXCE offspring when compared to Ctrl offspring (Fig. 2C; $P<0.05$).

Histological Analysis

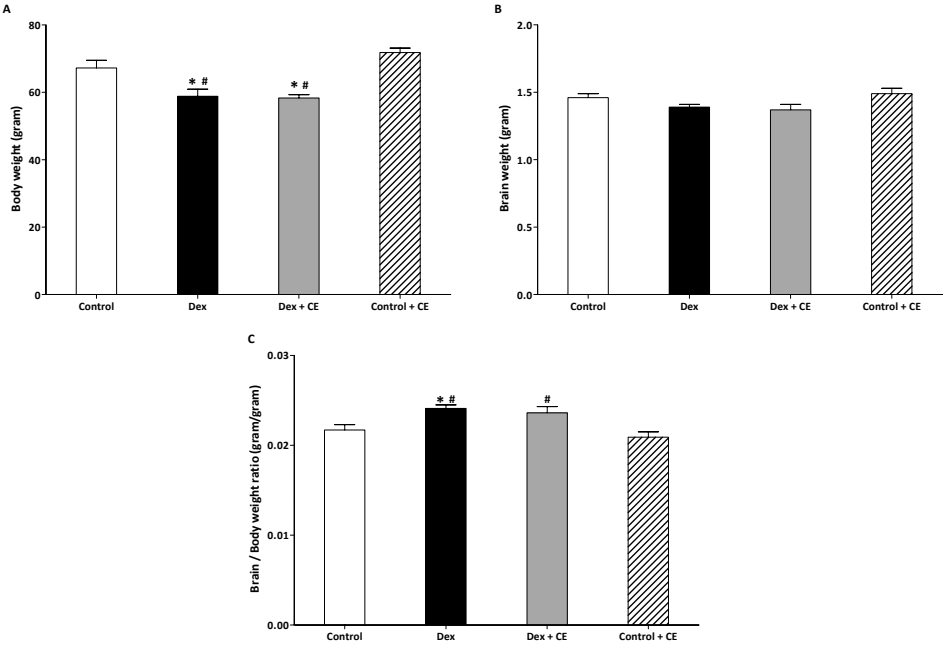
Gross morphology

Examination of the cresyl violet-stained coronal sections did not reveal any gross alterations in cytoarchitecture, cellular morphology, or anatomical organisation of the cortex or hippocampus between the groups at P22.

Regional brain volumes

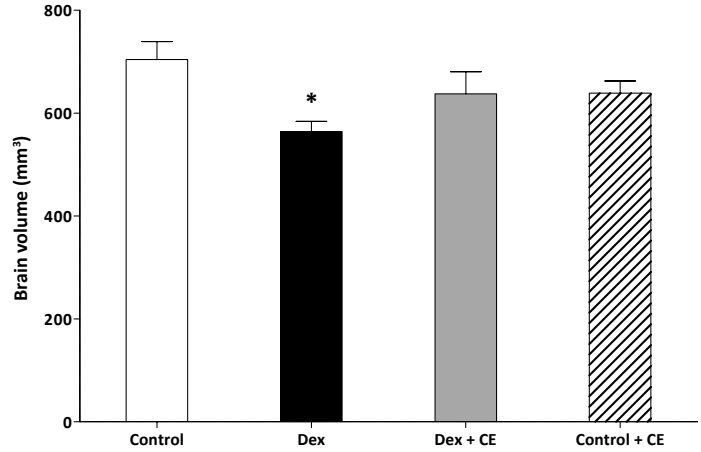
The volume of the cerebrum was significantly decreased in DEX offspring (Fig. 3; $P<0.05$). Dexamethasone in combination with vitamins did not significantly alter total brain volume ($P<0.05$). No further alterations in absolute regional volumes were observed between the groups (data not shown; $P>0.05$). Absolute regional volumes and regional volumes relative to total brain volume, were also unaltered (data not shown; $P>0.05$).

Figure 2. Body, brain and brain: body weight.



(A) Body weight, (B) brain weight and (C) brain: body weight at postnatal day 22 in control (Ctrl, n=5), dexamethasone (DEX, n=5), dexamethasone with vitamins C and E (DEXCE, n=5), and control with vitamins C and E (CtrlCE, n=5) pups. *, P<0.05 *versus* Ctrl; #, P<0.05 *versus* CtrlCE (One-Way ANOVA + Student-Newman-Keuls).

Figure 3. Cerebrum brain volume.

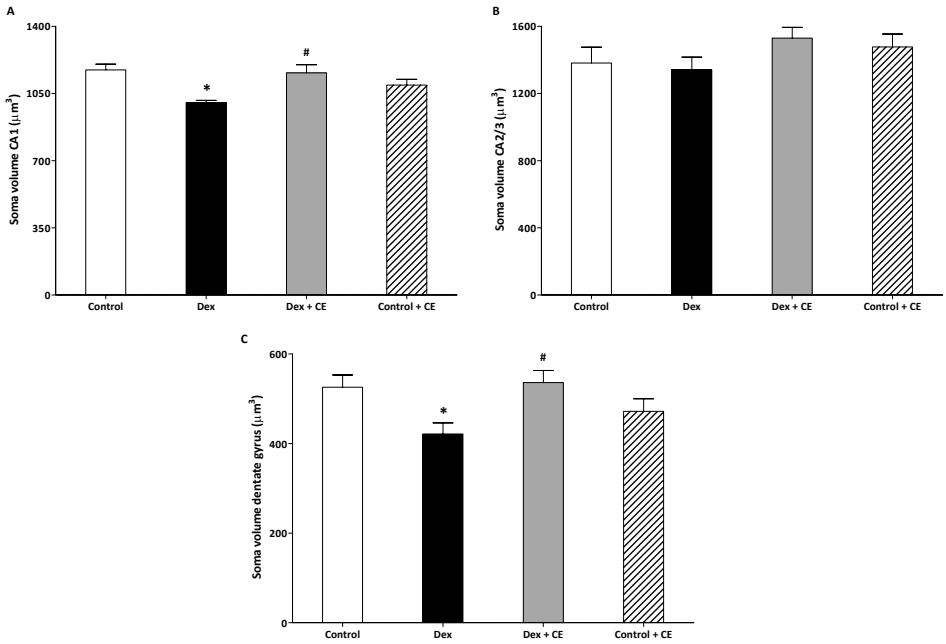


Brain volume of the cerebrum at postnatal day 22 in control (Ctrl, n=5), dexamethasone (DEX, n=5), dexamethasone with vitamins C and E (DEXCE, n=5), and control with vitamins C and E (CtrlCE, n=5) pups. *, P<0.05 *versus* Ctrl (One-Way ANOVA + Student-Newman-Keuls).

Neuronal numbers and soma volumes

There were no differences between the groups in neuronal numbers in either the cortex or hippocampus (Table 1, P>0.05). However, in DEX-treated offspring compared to control offspring, soma volume was significantly reduced in both the CA1 region and the dentate gyrus of the hippocampus (Fig. 4 A, C; P<0.05). Soma volume was not different between the groups in the CA2/3 region of the hippocampus (Fig. 4B; P>0.05). Soma volumes in the CA1 and dentate gyrus were unaltered in DEXCE offspring (P<0.05).

Figure 4. Soma volumes in the CA1 (A), CA2/3 (B), and dentate gyrus (C) of the hippocampus.



Soma volumes in the hippocampus are shown from the control (Ctrl, n=5), dexamethasone (DEX, n=5), dexamethasone with vitamins C and E (DEXCE, n=5), and control with vitamins C and E (CtrlCE, n=5) groups. *, P<0.05 *versus Ctrl*; #, P<0.05 *versus* DEX (One-Way ANOVA + Student-Newman-Keuls).

Table 1. Neuronal numbers in the cortex and hippocampus.

	Ctrl	DEX	DEXCE	CtrlCE
Neuronal Number in Cortex (million)	20.4±1.1	19.0±0.6	18.3±0.6	20.9±10.7
Cortical Reference Volume (mm^3)	343.0±14.4	293.8±9.6	335.8±26.6	321.0±16.7
Neuronal Number in CA1 (x1000)	410.1±24.5	413.8±17.7	464.4±28.4	452.6±14.8
CA1 Reference Volume (mm^3)	0.79±0.01	0.81±0.07	0.86±0.07	0.92±0.07
Neuronal Number in CA2/3 (x1000)	440.8±28.2	438.6±34.2	400.8±23.7	416.3±33.1
CA2/3 Reference Volume (mm^3)	0.73±0.10	0.81±0.02	0.75±0.05	0.81±0.10
Neuronal Number in Dentate Gyrus (x1000)	870.0±69.1	932.5±47.7	887.2±54.1	868.9±94.7
Dentate Gyrus Reference Volume (mm^3)	0.64±0.04	0.69±0.04	0.76±0.04	0.78±0.08

Neuronal numbers and reference volumes in the control (Ctrl, n=5), dexamethasone (DEX, n=5), dexamethasone with vitamins C and E (DEXCE, n=5) and control with vitamins C and E (CtrlCE, n=5) pups. The reference volumes are the volumes of the cortex and compartments of the hippocampal formation (CA1, CA2/3 and dentate gyrus) that neuronal number and soma volumes were measured in.

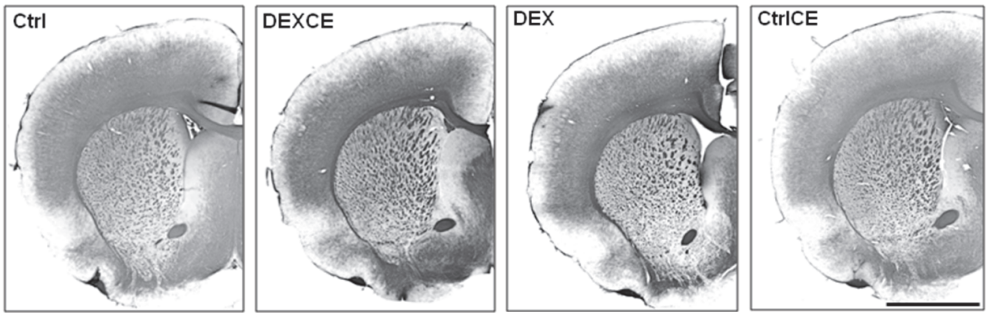
Myelination

The OD of MBP-stained fibres was significantly increased in the corpus callosum of DEXCE offspring when compared to Ctrl offspring (Ctrl: 0.211±0.006; DEX: 0.251±0.029 DEXCE: 0.299±0.021; CtrlCE: 0.259±0.016, Fig. 5; P<0.05). There were no significant differences between the groups in relation to the OD of MBP-stained fibres in the cortex (Ctrl: 0.184±0.008; DEX: 0.176±0.024; DEXCE: 0.204±0.014; CtrlCE: 0.185±0.01; P>0.05), or the extent of myelination (Ctrl: 58.9±0.8%; DEX: 58.9±0.8%; DEXCE: 56.5±1.0%; CtrlCE: 61.9±2.3%; P>0.05).

Western blot analysis

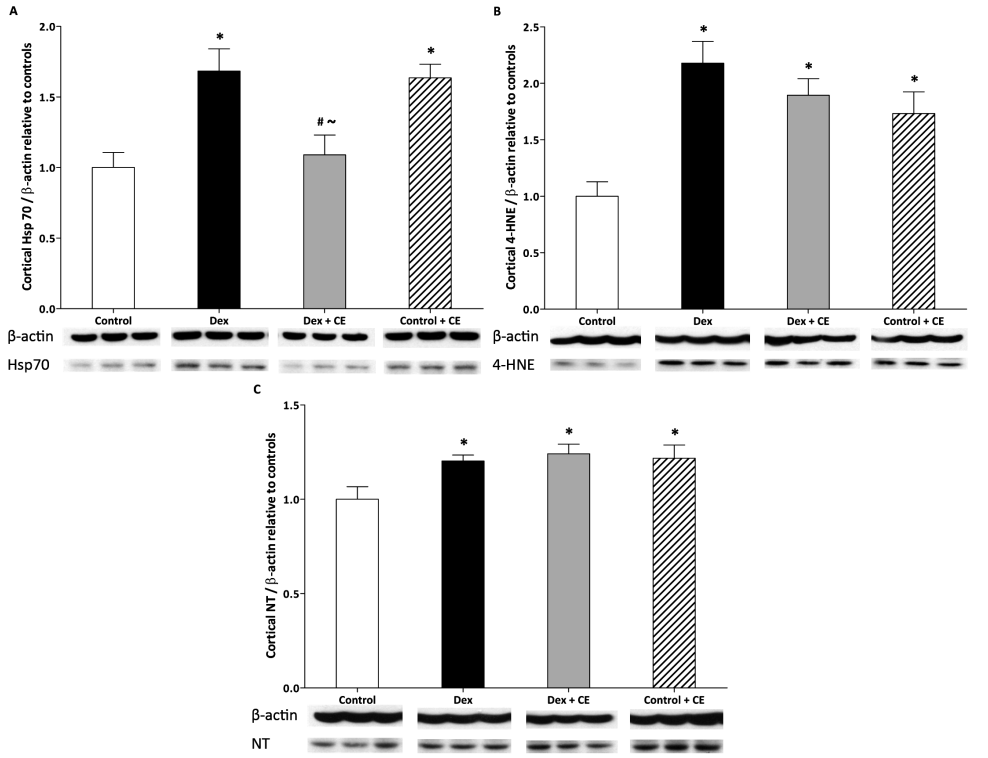
At P21, DEX increased the protein expression in the cortex of three established molecular indices of oxidative stress: Hsp70 (+68%), 4-HNE (+118), and NT (+20%, Fig 6A-C; P<0.05). Dexamethasone in combination with vitamins did not significantly alter Hsp70 protein expression relative to control or DEX-treated offspring (+9%; P<0.05). However, 4-HNE (+95%) and NT (+24%) remained up-regulated in the cortex (P<0.05). Relative to control offspring, no significant alterations in protein expression were observed in the hippocampus of DEX (Hsp70: +4%; 4-HNE: -6%; NT: -5%, data not shown; P>0.05) or DEXCE offspring (Hsp70: -21%; 4-HNE: -1% ; NT: -28%, data not shown; P>0.05). Treatment of neonates with vitamins alone increased Hsp70 (+63%) and 4-HNE (+73%) in the cortex (Fig. 6 A, B; P<0.05,), and NT in both the cortex (+22%, Fig. 6C; P<0.05) and hippocampus (+35%, data not shown; P<0.05).

Figure 5. Photomicrograph of myelin staining.



Coronal section of control (Ctrl), dexamethasone (DEX), dexamethasone with vitamins C and E (DEXCE), and control with vitamins C and E (CtrlCE)-treated offspring stained with myelin basic protein (MBP). Overall, the optical density (OD) of myelin staining in the corpus callosum was significantly increased in DEXCE offspring when compared to Ctrl offspring (P<0.05; One-Way ANOVA + Student-Newman-Keuls). Scale bar= 2.5mm.

Figure 6. Expression of heat-shock protein 70 (Hsp70, 70KDa, A), 4-hydroxynonenal (4-HNE, 54kDa, B), and nitrotyrosine (NT, 35kDa, C) in the cortex.



Representative Western blots are shown from the control (Ctrl, n=6), dexamethasone (DEX, n=6), dexamethasone with vitamins C and E (DEX CE, n=6), and control with vitamins C and E (CtrlCE, n=6) groups. Expression of the proteins was quantified and expressed as a ratio to β-actin. *, P<0.05 *versus* Ctrl; #, P<0.05 *versus* DEX; ~, P<0.05 *versus* CtrlCE (One-Way ANOVA + Student-Newman-Keuls).

Discussion

The data show that postnatal treatment with dexamethasone in human clinically-relevant doses decreased total brain volume and reduced neuronal soma volume in the CA1 and dentate gyrus of the hippocampus. The study adds the novel observation that co-administration of antioxidant vitamins with dexamethasone improved total brain volume and neuronal soma volumes in the hippocampus. Postnatal treatment with dexamethasone also increased three molecular indices of oxidative stress in the cortex. Co-administration of antioxidant vitamins restored Hsp70 protein expression in the cortex, however the expression of 4-HNE and NT remained significantly elevated. Treatment of neonates with vitamins alone induced oxidative stress in the cortex.

In the present study, morphological analysis of brain tissue revealed that volume of the cerebrum was significantly decreased in dexamethasone-treated offspring when compared to controls. Importantly, co-administration of dexamethasone with antioxidant vitamins prevented this effect. As there were no regional alterations in brain volumes when expressed relative to total brain volume, it appears that the brains of the dexamethasone-treated offspring were proportionally smaller. A decrease in whole cerebral tissue volume may be explained by reductions in neuronal numbers, soma volumes and/or delayed dendritic growth. To explore these possibilities, total neuronal numbers and soma volumes were assessed in the cortex and hippocampus, respectively. Total neuronal number was not altered following dexamethasone treatment in either the cortex or hippocampus. However, in dexamethasone-treated offspring, soma volume was reduced in the CA1 and the dentate gyrus of the hippocampus. Smaller neuronal volumes may be related to a decline in cellular function [42], selective loss of larger neurons, which has been observed in neurodegenerative diseases [43], or reflect neuronal shrinkage, which often precedes neurodegeneration [44]. Dexamethasone may delay neuronal differentiation or dendritic growth [13], which, in addition to the reduction in soma volumes, may also account for the reduction in brain volume. Antioxidant vitamin supplementation in dexamethasone-treated offspring prevented the decrease in hippocampal soma volumes. The data provide evidence for the powerful neuroprotective effects of vitamins C and E following glucocorticoid therapy.

To explore the mechanisms via which clinically-relevant doses of dexamethasone affected cerebral volume and the beneficial effects of vitamins C and E, the present study focussed on three established molecular indices of oxidative stress known to be affected by glucocorticoid treatment. In humans, corticosteroid therapy has been reported to markedly increase levels of 4-HNE [45] and NT immunoreactivity [22]. In addition, it is established that dexamethasone decreases eNOS protein and/or mRNA expression in several tissues in the rat [19,46,47]. Dexamethasone decreases eNOS activity, largely by limiting substrate availability, thereby promoting eNOS uncoupling leading to further superoxide generation and reduced NO bioavailability [19,21,46,48]. In the present study, dexamethasone increased the expression of Hsp70, 4-HNE, and NT in the cortex. Vitamin administration attenuated the increase in Hsp70 protein expression induced by dexamethasone treatment; however, the up-regulation of 4-HNE and NT remained unchanged. Similarly, previous studies have reported that vitamin C supplementation attenuated the increase in Hsp70 expression in muscle following physiological oxidative stress induced by exercise [49].

Vitamin C and E supplementation in combination could also decrease Hsp70 expression in the brain following heat stress [50]. Data in the present study therefore confirm that clinically-relevant doses of dexamethasone induce oxidative stress in the developing brain and that antioxidant therapy partially protect against this effect. The lack of demonstrable structural and neuronal injury, particularly in the cortex where oxidative stress was evident, may not necessarily imply normal function following postnatal glucocorticoid therapy. For instance, oxidative or nitrosative stressed neurons, injured by free radicals, might be present physically but be non-functional [51,52]. Further, clinical evidence does suggest that early postnatal dexamethasone therapy can have long-term adverse neuromotor and cognitive outcomes [8,9].

The physiology underlying the inhibitory effects of antioxidant vitamins on the expression of Hsp70 in past and present studies is likely multi-factorial and these mechanisms may also relate to the protective effect of antioxidants against glucocorticoid-induced reduction in cerebral volume, as shown in the present study. For instance, in addition to quenching ROS directly [38], antioxidant vitamins can act through tetrahydrobiopterin (BH4) to stimulate NO production by endothelial NO synthase [53]. Antioxidant vitamins can also prevent the S-nitrosylation of sensitive thiol groups on endothelial NO synthase, thereby maintaining their stability and the affinity of NO synthase for BH4 [54]. Further, it has been reported that vitamin C may enhance the expression and/or action of potent antioxidant enzymes [55]. Since manipulation of the vascular NO:$^{\bullet}O_2^-$ ratio is an important determinant of vascular tone, whereby a decrease in the ratio promotes vasoconstriction and an increase leads to vasodilatation [56], glucocorticoid-induced oxidative stress will favour a decrease in perfusion and antioxidant therapy will oppose this. Accordingly, several studies have shown that clinically-relevant dosing regimens of glucocorticoids in perinatal practice increase vascular resistance in several vascular beds, including the cerebral circulation [57-59]. Any effects of antioxidant vitamins which enhance the bioavailability of NO will favour maintained perfusion and thereby a protection against the stunting effects of glucocorticoids on growth, particularly on circulations which are highly dependent of NO, such as the cerebral vascular bed.

The mechanisms underlying the region-specific pattern of cerebral oxidative stress reported in the present paper are not well understood. One explanation may be that the neuronal populations in the cortex show greater vulnerability to oxidative stress as a result of a greater oxidative burden and/or lower antioxidant protection. Alternatively, it is possible that the hippocampus may have shown early changes in markers of oxidative stress following postnatal glucocorticoid treatment, but that these indices were below the threshold of detection with available techniques or that they had subsided by the time of tissue collection.

Additional data in the present study show that treatment of neonates with vitamins alone increased Hsp70 and 4-HNE in the cortex, and NT in both the cortex and hippocampus. Although antioxidant vitamin supplementation may improve disease states and/or conditions associated with enhanced oxidative stress, antioxidant treatment in healthy conditions where the physiology of the individual is already replenished with an appropriate equilibrium of pro- and anti-oxidant mechanisms, may, in fact, lead to excess NO bioavailability. The latter will promote peroxynitrite generation, thereby mimicking oxidative stress with subsequent induction of a number of the unwanted side-effects [60,61].

Postnatal dexamethasone treatment, with or without vitamins, significantly reduced body weight at P22. Maternal antenatal treatment with glucocorticoids has been shown to reduce weight gain in human infants [62] and suppress growth until weaning in rats [63]. Postnatally, glucocorticoids reduce somatic growth in premature human infants [64]. Our results also confirm similar findings reported in other experimental studies [35,36,65-67]. This reduction in somatic weight is likely due to the well-described direct effects of glucocorticoids on overall tissue accretion and catabolism [68-70] rather than the effects of glucocorticoid-induced oxidative stress on perfusion of regional circulations. Dexamethasone appeared to have a greater effect on body weight than brain weight (brain sparing), which suggests an increase in vascular resistance in peripheral circulations. The peripheral vasoconstrictor effects of dexamethasone in early life are well known. Two separate studies have reported that treatment of fetal sheep in late gestation with glucocorticoids leads to an increase in vascular resistance in peripheral circulations [57,58]. In this study, dexamethasone treatment reduced survival to 70%, a finding supported by other studies in neonatal rat pups [71-73]. This increase in mortality may be attributed to an inability of the offspring to utilise nutrients, which may be due to decreased suckling and/or accelerated gut closure [74].

In conclusion, postnatal glucocorticoid treatment in human clinically relevant doses reduced total brain volume and soma volumes in the hippocampus and induced oxidative stress in the developing brain. Combined glucocorticoid and antioxidant treatment may ameliorate some of the adverse consequences of postnatal glucocorticoid therapy on the developing brain. However, antioxidant vitamin supplementation is not recommended in healthy offspring. The data provide the proof of concept that antioxidant therapy may antagonise some of the unwanted effects of potent glucocorticoids needed in clinical practice. It is now important to investigate the value of this combined therapy on cerebral structure and functional development in an experimental model that closely mimics conditions associated with premature birth in humans, including established chronic lung disease or bronchopulmonary dysplasia.

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Chapter 5

Statins prevent adverse effects of postnatal glucocorticoid therapy on the developing brain in rats

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Abstract

Background

Postnatal glucocorticoid therapy in the treatment of chronic lung disease benefits lung function, however it adversely affects brain development. We hypothesized that combined postnatal glucocorticoid and statin therapy diminishes adverse effects of glucocorticoids on the developing brain.

Methods

On postnatal days (P) 1-3, one male pup per litter received i.p. injections of saline (Control 'C', n=13) or dexamethasone (0.5, 0.3, 0.1 µg/g; D, n=13), ± pravastatin (10 mg/kg i.p; CP, n=12; DP, n=15). Statins or saline continued from P4-6. At P21, brains were perfusion fixed for histological and stereological analyses.

Results

Relative to controls, dexamethasone reduced total (837±23 vs. 723±37), cortical (378±12 vs. 329±15) and deep gray matter (329±12 vs. 284±15) volume (mm^3), cortical neuronal number (23±1 vs. 19±1 $x10^6$) and hippocampal neuronal soma volume (CA1: 1206±32 vs. 999±32; dentate gyrus: 679±28 vs. 542±24 μm^3; all $P<0.05$. Dexamethasone increased the GFAP-positive astrocyte density in the white matter (96±2 vs. 110±4 /$0.1mm^2$); $P<0.05$. These effects no longer occurred in brains from pups treated with combined dexamethasone and pravastatin. Pravastatin alone had no effect on these variables.

Conclusion

Concomitant dexamethasone with statins in premature infants may be safer for the developing brain than dexamethasone alone in the treatment of chronic lung disease.

Introduction

Chronic lung disease (CLD) is a common outcome in extremely preterm neonates with severe respiratory distress syndrome and carries a high incidence of morbidity and mortality [1]. Glucocorticoids, predominantly dexamethasone, are used to decrease inflammatory responses, accelerate surfactant production and lung maturation, thereby improving respiratory function [2].

Despite the well-established beneficial effects of postnatal glucocorticoid therapy, there has been serious growing concern regarding their clinical use because of unwanted side effects on somatic growth and weight gain in premature babies [3,4]. Accumulating evidence from clinical trials has also demonstrated an association between postnatal steroid therapy and adverse neuromotor and cognitive outcomes [4,5]. Furthermore, experimental studies in neonatal animals have demonstrated adverse effects of potent glucocorticoids, such as dexamethasone, on brain growth, cell division, differentiation, myelination, apoptosis, and neurogenesis [6-8].

To date, the mechanism underlying the unwanted side effects of glucocorticoid therapy on the developing brain remains unknown, preventing the identification of plausible modified therapies to maintain the beneficial, but prevent the adverse, effects of steroid treatment. Glucocorticoids are known to decrease nitric oxide (NO) bioavailability and increases in NO via antioxidant treatment have been shown to prevent or partially restore glucocorticoid-induced cardiovascular dysfunction and hypertension [9,10], implying impaired NO physiology as the culprit mechanism mediating unwanted side-effects of glucocorticoids. Recent evidence indicates that statins, which are widely prescribed drugs for the primary prevention of coronary heart disease [11], have additional beneficial actions beyond that of cholesterol reduction via increasing NO bioavailability. Indeed, clinical and experimental evidence indicates that these pleiotropic effects of statins, by improving endothelial function, might be potentially useful for the treatment of neurologic disorders, such as ischemic stroke, Parkinson's disease, Alzheimer's disease and vascular dementia [12-14]. However, whether statins can protect the developing brain has never been investigated. Using an established experimental model of prematurity and postnatal glucocorticoid therapy [10,15,16], this study tested in rats the hypothesis that combined postnatal glucocorticoid and statin therapy will diminish the adverse effects of glucocorticoids on the developing brain by preserving NO bioavailability.

Materials and Methods

Ethical Approval

The study was approved by the Cambridge University Ethical Review Committee. All procedures were carried out under the UK Animals (Scientific Procedures) 1986 Act and conducted under the authority of the appropriate project license.

Animals and Experimental Design

Fifty three pregnant Wistar rats (Charles River, Margate, UK) with timed gestations were individually housed (23±1°C, light:dark, 12:12 hour) with access to food (Special Diet Services, Essex, UK) and water. All dams delivered on day 22 of gestation, P0. Litters were divided into four groups: control 'C', n=13; dexamethasone 'D', n=13; dexamethasone with pravastatin 'DP', n=15, and control with pravastatin 'CP', n=12.

Within 3-5 hours of birth, pups were sexed by measurement of ano-genital distance and weighed, and litters reduced to 8 pups per dam (four males and four females) to standardize postnatal nutrition and maternal care. To account for sex differences, only male pups within each litter received treatment (C, n=52; D, n=52; DP, n=60; and CP, n=48). C pups received injections of saline from P1-P6. D pups received a tapering course of dexamethasone (Dexamethasone-21-phosphate, disodium salt, Sigma-Aldrich, Dorset, UK: 0.5, 0.3, and 0.1 $\mu g.g^{-1}.day^{-1}$) from P1-P3 and saline from P1-P6. DP pups received the same tapering course of dexamethasone from P1-P3 with additional pravastatin (Sigma, Dorset, UK: 10 $mg.kg^{-1}$) from P1-P6. CP pups received pravastatin from P1-P6. Statin treatment was extended for an extra three days post glucocorticoid administration to counteract any possible lag in the effects of dexamethasone. The volume of each injection was 10 $\mu l.g^{-1}$. The treatment protocol for dexamethasone was proportional to a 21 d tapering course of dexamethasone used in human preterm infants to prevent or diminish chronic lung disease [16]. The dose of pravastatin represents an intermediate level between clinical studies and doses used in previous animal experiments [17,18]. Body weight was recorded from P0 to P7 and every other day thereafter.

Tissue Collection and Histological Analysis

At P21, some of the pups from each litter (C, n=8; D, n=7; DP, n=8; CP, n=7) were deeply anaesthetized (0.2 ml total volume, i.p., 100 $mg.ml^{-1}$ ketamine, Fort Dodge Animal Health, Southampton, UK and 20 $mg.ml^{-1}$ xylazine, Millpledge Veterinary, Nottinghamshire, UK) and perfused intracardially with NaCl followed by 4% paraformaldehyde (PFA).

The cerebrum was exhaustively sectioned at 50 µm (RM 2235 vibratome, Leica Microsystems, Wetzlar, Germany). Quantification of the brain tissue was performed with an Olympus BX-50 microscope and CAST grid (Olympus, Southend-on-Sea, UK), with the observer being blind to the treatment group. Sections were washed for 30 minutes in phosphate buffered saline (PBS, Sigma-Aldrich, Dorset, UK), incubated with 30% H_2O_2 for five minutes to block endogenous peroxidase activity and washed in PBS for 30 minutes. Subsequently non-specific binding was blocked with 4% bovine serum albumin (BSA) in PBS for 10 minutes. Sections were then incubated with primary antibody (Myelin basic protein (MBP); 1:400, Millipore, Watford, UK; Neuronal Nuclei (NeuN), 1:400, Millipore, Watford, UK; Glial fibrillary acidic protein (GFAP) 1:500, Millipore, Watford, UK) in primary diluent (2% BSA in PBS containing 0.3% Triton; Sigma Aldrich, Dorset, UK) overnight. The following day sections were washed 30 minutes in PBS, incubated for one hour with secondary antibody (1:400, Vector Laboratories, Burlingame, California) in secondary diluent (2% BSA in PBS) then washed for 15 minutes in PBS. Sections were incubated for one hour in Avidin Biotin (AB) (Vector Laboratories, Peterborough, UK) in PBS then washed in PBS for 15 minutes. Staining was visualized by adding metal DAB (Thermo Scientific, Rockford, Illinois)

in peroxide buffer (Thermo Scientific, Rockford, Illinois) for two minutes to the sections. Tissue was then mounted with 0.5% gelatin in PBS on slides. For lectin staining, the same procedure was followed as for the immunohistochemistry methods, except that the secondary antibody was omitted (1:200, Vector Laboratories, Burlingame, UK).

Volumetric analysis
Systematic random sampling [19] was used to select, without bias, 10 sections per animal for analysis. Selected sections were stained using 1% Cresyl Violet. A point grid was superimposed on the sections and viewed using a x1.25 objective to assess the volume of the cerebral hemispheres, cortex, white matter, and hippocampus. Points falling on each compartment were counted and the Cavalieri principle [19] was applied in order to calculate estimated volumes:

$$V_{(obj)} = t \times \Sigma a = t \times a_{(p)} \times \Sigma P$$

where $V_{(obj)}$ represents the estimated volume of the brain region, t is the total length of the brain (t = no. of sections x section thickness), $a_{(p)}$ is the area associated with each point and ΣP is the sum of points for that formation.

Neuronal number and soma volume quantification using the fractionator method
Neuronal nuclei antigen-stained stained sections containing the cortex and hippocampus (divided into the CA1, CA2/3 and dentate gyrus) were sampled. Step motors on the microscope were used to randomly sample a known fraction of the tissue. An unbiased counting frame of known dimensions was superimposed on the tissue image. Nuclei within the counting frame, or those touching the permitted lines of the counting frame, were counted. To calculate total neuronal number, the following formula was applied:

$$\text{est } N = \Sigma \text{ n} \,.\, f_1 .\, f_2$$

where N is the estimated total number of nuclei, Σ n is the sum of nuclei counted in the brain sample, f_1 is the reciprocal of the sampling fraction, and f_2 is the areal sampling fraction.

The soma volume of neurons from the cortex and hippocampus were calculated using the vertical dissector tool in the CAST grid programme [15]. Soma volume was calculated using the following formula:

$$Vn = 4\pi/3 * I3n$$

where I is the intercept measurements.

Myelination
The optical density of myelin basic protein-stained fibers was measured in the corpus callosum and cortex using ImageJ (V1.38u, National Institutes of Health, Bethesda, Maryland). Eight fields within the corpus callosum and cortex were examined over three sections per animal. Optical

density was measured as gray levels. Non-specific background optical densities were measured at each level in a region devoid of myelin basic protein-immunostaining and these were subtracted from the corpus callosum and cortex values. The area of myelin basic protein-positive fibers in the cortex, relative to cortical size, was assessed using the same software.

Quantification of GFAP-positive astrocytes
Quantitative analysis of astrocytes was performed using GFAP. Areal density of GFAP positive astrocytes was assessed in the white matter of the cingulum (see plate 15-36 in Paxinos & Watson [20]). Labelled cells were counted at x400 magnification over eight fields per animal (2 per section); values for each animal were pooled and a mean value was calculated. A mean of means ± SEM was calculated for each group.

Volume fraction of blood vessels
The volume fraction of the parenchyma occupied by blood vessels was assessed in the cortex and hipppcampus using lectin-stained sections. A point grid was superimposed on the sections and viewed at x200 magnification, and the percentage of blood vessels assessed by counting the number of intersections in up to 25 fields in both the cortex and hippocampus.
Fields were randomly sampled in the primary and secondary motor cortex and the primary somatosensory cortex, in both hemispheres over 10-12 sections. Fields in the hippocampus were taken from the stratum lacunosum-moleculare and stratum radiatum, in both hemispheres over 3 sections.

NOx assay
Plasma nitrite (NO_2^-) and nitrate (NO_3^-) concentrations were determined at P21 in a subgroup of the pups (C: n= 10; D: n=6; DP: n=6; CP: n= 9). Levels of NOx were measured from plasma samples using the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, Michigan) based on the principles of the Griess color reaction [21]. NOx was measured from plasma samples, which were ultra-filtrated to reduce background interference due to hemoglobin. Assay buffer, nitrate standards and 40 µl of plasma samples were loaded in duplicate into a 96 well microplate with nitrate reductase and an enzyme cofactor. The plates were allowed to incubate for 60 minutes at room temperature. Before a further 10 minutes incubation, Griess reagents were added to the wells. Following this, absorbance was read at 540 nm (Biotek ELx800 Absorbance Microplate Reader, Potton, UK). Plasma NO_2^- levels were measured using the same method but omitting the nitrate reductase step. Plasma NO_3^- levels were calculated as total NOx minus NO_2^-. Intra-assay variability was 2.7%, whereas inter-assay variability was 3.4%. The lower limit of detection was 0.24 µM.

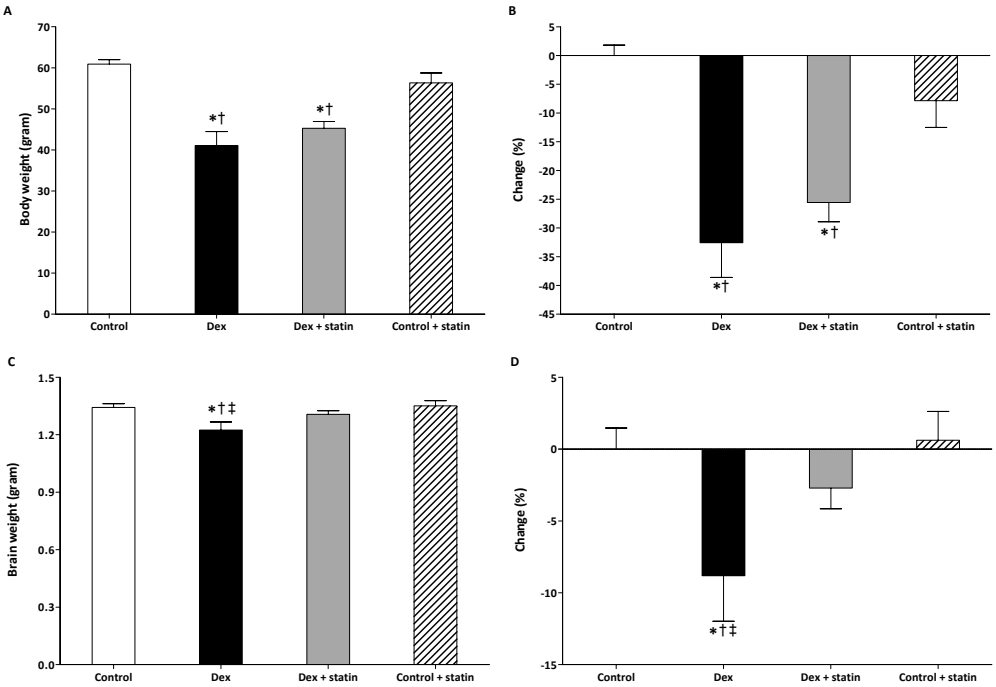
Statistical Analysis
Data are presented as mean ± SEM unless otherwise indicated. Data were analysed by one-Way ANOVA followed by the Student-Newman-Keuls *post hoc* test. SigmaStat software (SigmaStat for Windows, Version 2.0) was used for all statistical analyses. P values < 0.05 were accepted as statistically significant.

Results

Body and brain weights

Compared to control offspring, dexamethasone significantly reduced body and brain weight at postnatal day 21 (P21), whether expressed as absolute weight or as a percentage of control weight (Figure 1A-D; P<0.05). Postnatal treatment with combined dexamethasone and pravastatin restored brain weight to control levels, however body weight remained reduced. Postnatal treatment of control animals with pravastatin alone had no effect on body or brain weight (Figure 1A-D).

Figure 1. Effects on body and brain weight



(A) Total body weight, (B) percentage change from control body weight, (C) total brain weight, (D) percentage change from control brain weight at postnatal day 21 in control (n=8), dexamethasone (n=7), dexamethasone with pravastatin (n=8) and control with pravastatin (n=7). * P<0.05 versus control; † P<0.05 versus control with pravastatin; ‡ P<0.05 versus dexamethasone with pravastatin (One-Way ANOVA + Student-Newman-Keuls).

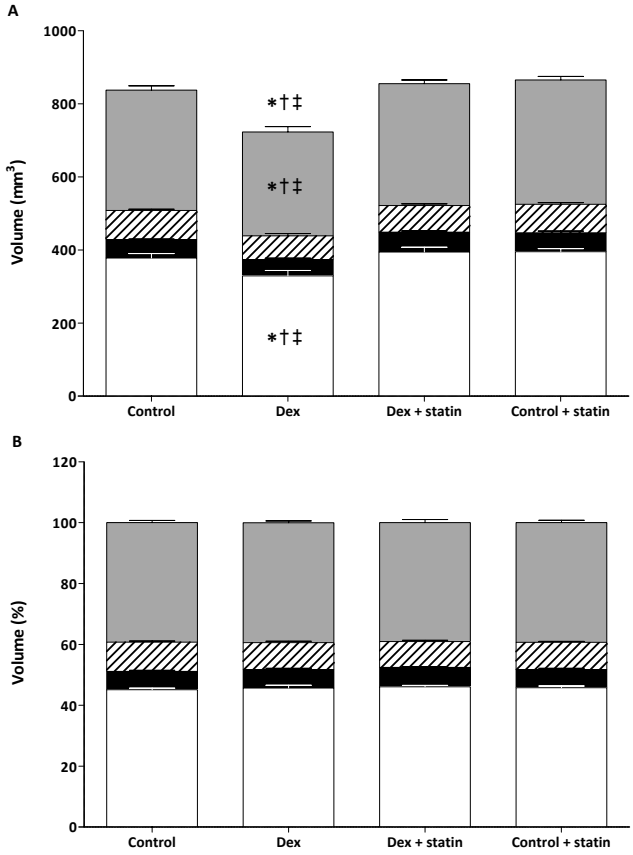
Cerebral histological analysis

Gross morphology and regional brain volumes

At P21, examination of the cresyl violet-stained coronal sections did not reveal any gross alterations in cytoarchitecture, cellular morphology, or anatomical organisation of the cortex or hippocampus

between the four experimental groups. However, relative to control animals, postnatal treatment with dexamethasone reduced absolute cerebral volume; this decrease appeared to be due to significant reductions in cortical and deep gray matter volumes (Figure 2A; P<0.05). Concomitant postnatal treatment with pravastatin restored total cerebral, cortical and deep gray matter volumes to control levels. No significant alterations in hippocampal volumes or white matter volumes were observed between the groups. Postnatal treatment with pravastatin alone had no effect on absolute or regional brain volumes (Figure 2A). Postnatal dexamethasone treatment reduced brain volume proportionally as differences disappeared when comparing compartmental volumes expressed relative to total cerebral volume (Figure 2B).

Figure 2. Effects on brain volume



(A) Total brain volume and volumes of deep gray matter, hippocampus, white matter and cortex (B) deep gray matter volume, hippocampal volume, white matter volume and cortical volume as a percentage of total brain volume, at postnatal day 21 in control (n=8), dexamethasone (n=7), dexamethasone with pravastatin (n=8), and control with pravastatin (n=7) pups. Gray bar: deep gray matter, striped bar: hippocampus, black bar: white matter, white bar: cortex. * P<0.05 versus control; † P<0.05 versus control with pravastatin, ‡ P<0.05 versus dexamethasone with pravastatin (One-Way ANOVA + Student-Newman-Keuls).

Neuronal numbers and soma volumes

Compared to control offspring, neuronal number in the cortex was significantly reduced in P21 offspring following postnatal treatment with dexamethasone ($P<0.05$); soma volume of cortical neurons was unaltered (Table 1). In contrast, in the hippocampus, neuronal numbers were unaltered, however soma volumes were significantly reduced in the CA1 and the dentate gyrus of P21 pups treated with dexamethasone postnatally (Table 2; $P<0.05$). These alterations in neuronal number and soma volume no longer occurred in P21 pups treated with combined dexamethasone and pravastatin postnatally. Postnatal treatment with pravastatin alone had no effect on neuronal number or soma volume.

Table 1. Neuronal numbers and soma volumes in the cortex.

	C	D	DP	CP
Cortical Neuronal Number (x10^6)	22.8±1.05	18.9±0.78*	20.4±1.14	20.7±0.87
Average soma volume of cortical neurons (μm^3)	1176.4±21.1	1095.9±41.0	1140.7±43.9	1179.1±28.8

Neuronal numbers and soma volumes in the cortex of control (C, n=8), dexamethasone (D, n=7), dexamethasone with pravastatin (DP, n=6) and control with pravastatin (CP, n=6) pups. *p<0.05 versus C (One-Way ANOVA + Student-Newman-Keuls).

Table 2. Neuronal numbers and soma volumes in the hippocampus.

	C	D	DP	CP
Neuronal Number in CA1 (x1000)	408.6±16.5	400.4±23.4	415.1±23.2	416.3±10.0
Average soma volume of CA1 neurons (μm^3)	1205.5±32.4	999.3±32.3*†	1109.4±54.8	1178.5±79.1
Neuronal Number in CA2/3 (x1000)	416.8±29.1	349.9±33.2	360.6±38.5	446.0±26.5
Average soma volume of CA2/3 Neurons (μm^3)	1557.5±46.1	1381.8±93.5	1564.5±116.0	1575.5±66.2
Neuronal Number in Dentate Gyrus (x1000)	990.6±52.1	931.2±82.5	1070.0±48.6	874.4±57.1
Average soma volume Dentate Gyrus Neurons (μm^3)	678.6±27.8	542.3±24.4*	596.9±25.4	624.7±26.9

Neuronal numbers and soma volumes in the hippocampus of control (C, n=8), dexamethasone (D, n=7), dexamethasone with pravastatin (DP, n=6) and control with pravastatin (CP, n=6) pups. * P<0.05 versus C, † P<0.05 versus CP (One-Way ANOVA + Student-Newman-Keuls

Astrocyte density

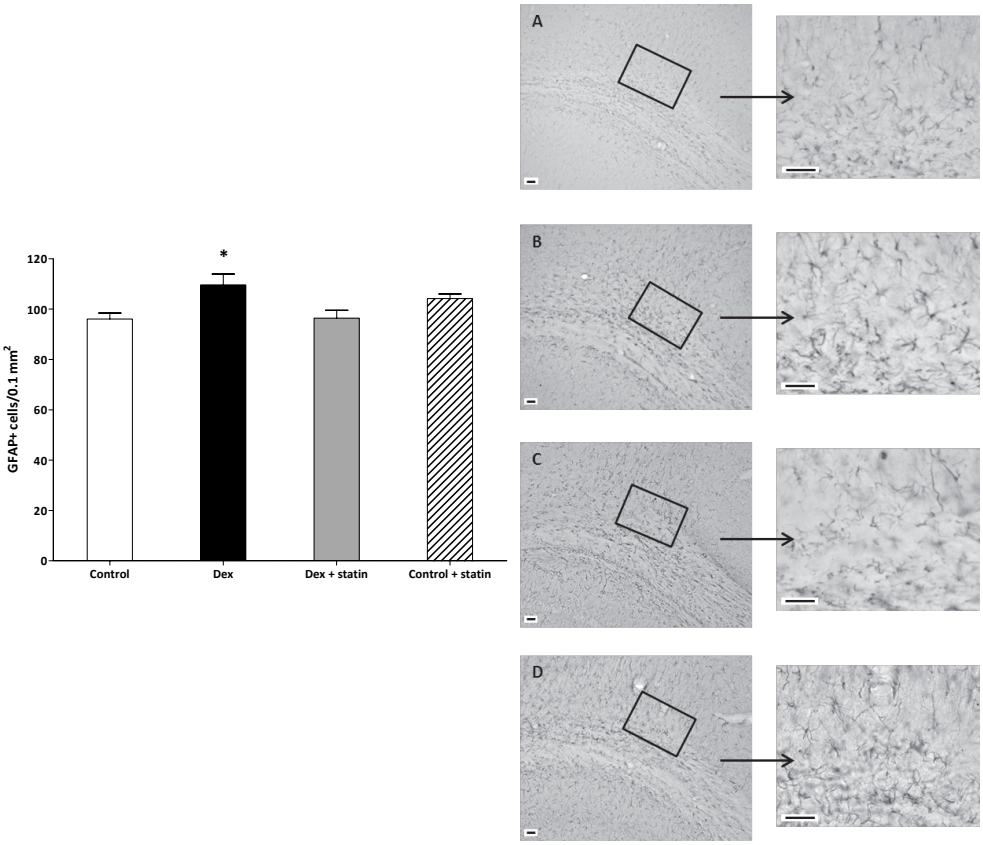
Postnatal treatment with dexamethasone significantly increased GFAP-positive astrocyte density in the cingulate white matter (Figure 3; $P<0.05$). Postnatal concomitant treatment of dexamethasone with pravastatin normalized GFAP-positive astrocyte density to control levels. Postnatal treatment with pravastatin alone had no effect on GFAP-positive astrocyte density (Figure 3).

Volume fraction of blood vessels and myelination

No differences in the volume fraction of blood vessels in the primary and secondary motor cortex, the primary somatosensory cortex, lateral and medial parietal association cortex and the primary and secondary visual cortex (C: 9.0±0.7%; D: 8.4±0.3%; DP: 7.2±0.2%; control with pravastatin: 7.9±0.9%; $P>0.05$) or in the stratum lacunosum-moleculare and stratum radiatum

of the hippocampus (C: 6.2±0.3%; D: 7.0±0.5%; DP: 6.0±0.4%; CP: 6.5±0.4%; P>0.05) were found between the four experimental groups. Similarly, no differences were found between the groups in relation to the optical density of myelin basic protein-stained fibers in the cortex (C: 0.39±0.03; D: 0.38±0.02; DP: 0.44±0.02; CP: 0.39±0.01; P>0.05), or the extent of myelination in the cortex (C: 73.1±0.6%; D: 70.0±1.4%; DP: 70.9±0.7%; CP: 72.4±0.8%; P>0.05). However, the optical density of myelin basic protein-stained fibers was significantly increased in the corpus callosum of P21 offspring treated postnatally with combined dexamethasone and pravastatin, but not pravastatin alone, when compared to control offspring (C: 0.49±0.04; D: 0.46±0.03; DP: 0.59±0.04; CP: 0.46±0.01; P<0.05).

Figure 3. Density of GFAP-positive astrocytes in the cingulate white matter



Density of GFAP-positive astrocytes in control (n=6), dexamethasone (n=6), dexamethasone with pravastatin (n=6) and control with pravastatin (n=6). * P<0.05 versus control (One-Way ANOVA + Student-Newman-Keuls).

Panels A-D, left: photomicrographs show in low power the area of the cingulate white matter where the number of GFAP-positive astrocytes were counted. A: control, B: dexamethasone, C: dexamethasone with pravastatin, D: control with pravastatin. Panels right: photomicrographs show the boxed areas in high power. All scale bars represent 30 µm.

NO bioavailability

Compared to controls, postnatal treatment with dexamethasone led to a significant decrease in plasma NOx, comprising plasma nitrite (NO_2^-) and nitrate (NO_3^-) (22.3±2.2 μM vs. 13.1±0.8 μM; $P<0.05$) at P21. In contrast, this decrease did not occur in pups following combined postnatal dexamethasone and pravastatin treatment (19.9±4.4 μM; $P<0.05$). Postnatal pravastatin treatment alone also significantly reduced plasma NOx levels (11.6±2.1 μM; $P<0.05$).

Discussion

The postnatal rat is an established experimental model of human prematurity, as postnatal development of neuronal, cardiovascular and respiratory function in this species compares with pre-natal milestones in the human [22,23]. Data in the present study show that postnatal treatment of rat pups with dexamethasone in human clinically-relevant doses decreased regional brain volumes, cortical neuronal number and hippocampal soma volume, while increasing the density of GFAP-positive astrocytes in the white matter when measured at weaning. Postnatal treatment with dexamethasone also led to a fall in plasma NOx concentrations at weaning. Further, the data show that postnatal combined treatment of dexamethasone with statins prevented the fall in plasma NOx concentrations and the adverse effects on cerebral development at P21. Treatment of newborn pups with pravastatin alone had no adverse effects on brain development but it reduced plasma NOx concentrations at weaning.

Stereological analysis of brain tissue in the present study revealed that the volumes of the cerebrum, cortex and deep gray matter were significantly decreased in dexamethasone-treated offspring. Brains of dexamethasone treated pups were proportionally smaller, as regional alterations were not observed when volumes were expressed as a percentage of total brain volume. The reduction in whole cerebral tissue volume and regional volumes induced by dexamethasone could be a result of a reduction in neuronal number, and/or soma volume and/or a decrease or delay in dendritic outgrowth. To differentiate between some of these possibilities we determined total neuronal number and soma volume in the cortex and hippocampus. The data revealed that cortical neuronal number, but not soma volume, was reduced following dexamethasone. However, the density of cortical neurons remained unaltered following dexamethasone treatment, indicating a reduction in neurons in proportion to the reduction in the size of the cortex. Dexamethasone-dependent reductions in cortical neuronal number have been previously described by Crochemore et al. [24] and may be due to a suppression of neurogenesis, as reported in tree shrews following psychosocial stress or following corticosterone treatment in rats [25,26], or due to impaired neuronal proliferation [8] or enhanced cell death [27], as also reported in rats following dexamethasone treatment. Additional data in our study show that soma volume, but not neuronal number, was reduced in the CA1 and the dentate gyrus of the hippocampus following postnatal dexamethasone treatment. The observed reduction in neuronal soma volume may be related to a decrease in cellular function [28], or reflect a delay in neuronal differentiation and/or dendritic growth, as previously described in the fetal baboon brain [6]. The decrease could further indicate

a preferential loss of larger neurons, which has been observed in neurodegenerative diseases [29], or reflect neuronal shrinkage, which often precedes neurodegeneration [30].

Beside the changes in gray matter we were also interested in white matter changes because of the association of dexamethasone with increased rates of cerebral palsy [2]. White matter damage is the leading pathological condition underlying cerebral palsy in children born prematurely and is characterized by an inflammatory response, astrogliosis, increased cell death and myelination impairment [31]. Data in the present study show that postnatal dexamethasone therapy was also associated with a significant increase in the density of GFAP-positive astrocytes in the cingulate white matter.

Results in the present study not only confirm the deleterious effects of postnatal dexamethasone administration on the developing brain, as reported in several species, but they also extend these findings to show that combined administration of postnatal dexamethasone with pravastatin prevented the decrease in regional brain volumes, cortical neuronal number and the reduction in hippocampal soma volume in the CA1 and the dentate gyrus. The group of 5-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) reductase inhibitors, or statins, have become one of the most effective and widely prescribed drugs for the primary and secondary prevention of cardiovascular disease [11]. In addition to their established lipid-lowering actions, positive effects of statins have been demonstrated on the cardiovascular system [32]; benefits which have all been credited to increases in NO bioavailability. To explore enhanced NO bioavailability as a potential mechanism mediating the protective effect of statins in dexamethasone treated pups, we measured plasma concentrations of NOx in the weanling rats. Plasma nitrite and nitrate (NOx) are stable end products of NO, providing a good first-order index on enhanced NO bioavailability. Glucocorticoid excess has been reported to enhance reactive oxygen species (ROS) generation directly [9], or secondary to endothelial nitric oxide synthase (eNOS) uncoupling by limiting the availability of co-factors, such as L-arginine and tetrahydrobiopterin (BH4) [33]. Oxygen free radicals, such as the superoxide anion, readily combine with NO, reducing its bioavailability [9]. Reduced NO bioavailability promotes increased vascular resistance and thereby a decrease in perfusion, including a reduction in blood flow in the cerebral vascular bed [34]. One mechanism by which statins may convey protection following dexamethasone treatment may therefore be by enhancing the bioavailability of NO to favor maintained perfusion, especially in highly NO-dependent circulations, such as the cerebral vascular bed. Statins have also been shown to promote phosphorylation of Akt [12]. Phosphorylation increases protein kinase activity, leading to Akt-mediated phosphorylation of eNOS, which stimulates NO production [35]. NO activates soluble guanylyl cyclase (sGC) leading to the formation of cGMP [12]. In the central nervous system, NO/cGMP signalling promotes synaptic plasticity, neurogenesis and angiogenesis [36,37] providing an additional pathway via which statin-induced increases in NO bioavailability may be protective on the developing brain.

Interestingly, data in the present study also show that treatment of postnatal rats with pravastatin alone did not have any adverse effects on any measured variable in the developing brain, but it did significantly reduce plasma NOx levels at P21. It is likely that the fall in plasma NOx following statin treatment alone is the result of negative feedback due to excess NO bioavailability and

thereby having no adverse effects on the brain. Chronic increases in NO have been reported to down-regulate sGC expression [38] and to increase phosphodiesterase (PDE) function [39], lowering the bioavailability of NO. This idea is consistent with dexamethasone-induced reductions in the concentration of the vasodilator gas with deleterious effects on the brain, versus effects of statins in dexamethasone-treated pups replenishing appropriate NO concentrations and therefore protecting the brain.

A final component of the present study shows that postnatal dexamethasone treatment significantly reduced body weight in the weanling pup. These data support similar findings reported in other experimental studies [15,16] and in premature human infants [3]. The deleterious effects on weight gain of dexamethasone are due to direct effects of glucocorticoids on tissue accretion and catabolism [40], explaining the lack of any protective effect on growth of co-administration with statins.

In conclusion, postnatal dexamethasone treatment in human clinically relevant doses reduced regional brain volumes, neuronal soma volumes in the hippocampus and increased the density of GFAP-positive astrocytes in the white matter of the developing brain. Combined postnatal treatment of dexamethasone with pravastatin prevented the adverse effects of dexamethasone on the developing brain. The data suggest that combined glucocorticoid and statin therapy may be safer in the preterm infant than glucocorticoid treatment alone.

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Chapter 6

Statin therapy prevents the detrimental effects of postnatal dexamethasone on the cardiovascular system

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Manuscript in preparation

Introduction

Premature delivery of the newborn is a significant cause of infant respiratory distress syndrome and is strongly associated with the development of bronchopulmonary dysplasia and chronic lung disease (CLD) secondary to the immature development of the lungs and clinical exposure to high levels of oxygen and mechanical ventilation [1]. The clinical use of glucocorticoids in premature neonates has become common practice following the pioneering work of Liggins who discovered that development of the fetal tissues in preparation for extra-uterine life was dependent upon a prepartum surge in fetal endogenous glucocorticoids and that exposure to synthetic glucocorticoids in premature individuals could aid pulmonary maturation [2,3]. It is now well established that pre- [4] and postnatal [5,6] glucocorticoids reduce the incidence of CLD and dependence on assisted ventilation in premature infants. Glucocorticoids improve respiratory function through alveolar maturation and induce the production of surfactant, which reduces respiratory work and increases functional residual capacity by promoting alveolar stability [7,8]. Further, they mediate their beneficial effects in part through their anti-inflammatory action [1]

Despite the well documented life-saving effects of perinatal glucocorticoid therapy on lung function, there is growing concern because serious and lasting adverse side effects have been reported in humans and in experimental animal models [6]. Glucocorticoid treatment stunts growth in humans [9] and in animal models [10-12]. Further, hypertension [5,11-13], vascular and endothelial dysfunction [14] and cardiac remodelling with dysfunction are all present [10,15,16]. Accumulating evidence suggests that one pathway by which glucocorticoids may promote their deleterious effects is through the inappropriate generation of ROS and decreased NO bioavailability [17,18]. For instance, glucocorticoids are known to activate pro-oxidant systems such as xanthine oxidase [18,19], and their adverse effects can be prevented by treatment with antioxidants such as tempol and vitamins C and E [14,20]. Given that ROS react with NO to increase vascular tone [17,21], it is possible that the adverse effects of glucocorticoids on the developing cardiovascular system are secondary to depleted bioavailability of NO [14,20].

A separate line of research shows that the group of HMG-CoA reductase inhibitors, or statins, have become one of the most effective and widely prescribed drugs for the primary and secondary prevention of cardiovascular disease [22]. In addition to their lipid lowering actions, so-called 'pleiotropic' actions of statins have been shown, including decreases in arterial stiffness [23], reductions in platelet aggregation [24] and improvements in vascular endothelial tone [25]. These benefits have all been credited to increases in NO bioavailability [26-28].

This study has intertwined these two lines of thinking to suggest that preterm infants treated with glucocorticoids may benefit from statin therapy in clinical practice. If true, glucocorticoids and statin therapy in perinatal medicine may ameliorate the detrimental effects of glucocorticoids on growth and the cardiovascular system whilst maintaining the beneficial maturational effects on the lung. This hypothesis was tested by investigating the effects of a human clinically-relevant dosing regimen of postnatal glucocorticoid therapy with and without pravastatin treatment on growth, cardiovascular function and pulmonary surfactant in the weanling rat.

Methods

Animals

All procedures involving animals were carried out under the Animals (Scientific Procedures) Act 1986 and approved by the Ethics Committee at the University of Cambridge, UK. Time mated pregnant Wistar rats (Charles River Limited, UK) were delivered to the University of Cambridge between 10-14 days of gestation and were individually housed under standard conditions (21±1^{o}C, 55% humidity, 12h/12h light/dark cycle) with free access to food and water. At birth (postnatal day 0, P0), pups from each litter were sexed, weighed and the litter size culled to eight (four males and four females chosen at random) to standardise feeding and maternal care, as previously described in detail [14,16]. Pups were sexed at birth by inspection by at least two observers.

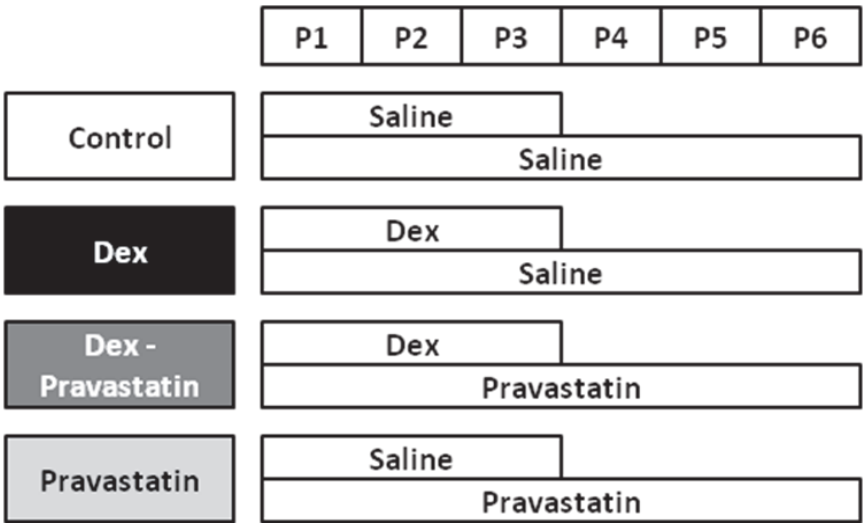
Experimental design

In this study, only male rat pups received treatment to control for sex differences. On postnatal days P1-P3, male pups received either saline (10 µl g^{-1} i.p.) or a three-day, tapering course of dexamethasone (10 µl g^{-1} i.p. of Dexamethasone disodium salt solution, Sigma-Aldrich, UK, in 0.9% saline equating to 0.5, 0.3, and 0.1 mg kg^{-1} d^{-1}; Figure 1). In addition, on postnatal days P1-P6 all male pups received either saline (10 µl g^{-1} i.p.) or pravastatin (10 mg kg^{-1} d^{-1} in 10 µl g^{-1} saline i.p. Pravastatin sodium, Sigma-Aldrich, UK). Therefore, the experimental design consisted of four treatment groups: Control (C), Dexamethasone (D), Dexamethasone + Pravastatin (DP) and Pravastatin (P) alone. Statin treatment was extended for an extra three days post glucocorticoid administration to counteract any possible lag in the generation of ROS. Pups were individually marked and weighed daily from P0 to P7 and every other day thereafter. Following treatment, pups remained with their mothers until P21. To control for within-litter variation, one pup per litter was used for any one outcome variable. Therefore, one male in each litter was used for: 1, *In vivo* cardiovascular assessment; 2, *In vitro* Langendorff isolated heart; 3, *In vitro* wire myography and 4, for measurement of plasma NO_x. Male pups only were used to control for any sex-specific effects of glucocorticoid treatment [14,16].

Biometry and blood sampling

In all animals following euthanasia at P21, body weight and crown rump length were measured to calculate ponderal index (weight/height^3). Head diameter, head length and foot length were also recorded. A blood sample was taken from animals that provided tissues for molecular biology analysis. In these animals and following *in vivo* experiments, the pup was dissected and the wet weight of the heart, brain, lung, liver and kidney were recorded. The right ventricle of the heart was dissected away from the left ventricle and septum and they were weighed individually. Tissues and plasma samples were stored at -80^{o}C until analysis.

Figure 1: Experimental Treatment protocol.



On postnatal days P1-P3 all male pups received either saline (10 µl g^{-1} i.p.) or a three-day, tapering course of dexamethasone (Dex; 10 µl g^{-1} i.p. of solution equating to 0.5, 0.3, and 0.1 mg kg^{-1} dissolved in saline i.p.). In addition, on post natal days P1-P6 all male pups received either saline or pravastatin (10 mg kg^{-1}, Pravastatin sodium, Sigma-Aldrich, UK).

NO_x assay

Plasma concentrations of NO_2^- and NO_3^- were determined by a commercially available Nitrate/Nitrite Colorimetric Assay Kit (Caymen Chemical, USA, Cat No. 780001) working on the principles of the Griess reaction [29]. In brief, total NO_x was measured from plasma samples which were ultra-filtrated to reduce the background interference due to any haemoglobin present. Assay buffer, nitrate standards and 40 µl of plasma samples were then loaded in duplicate into a 96 well microplate with an enzyme cofactor and nitrate reductase before a 60 min incubation at room temperature. Following further addition of the Griess reagents, the plates were allowed to incubate for 10 minutes before reading absorbance at 540nm (Bioteck ELx800 Absorbance Microplate Reader). Plasma NO_2^- concentrations were measured by the same method but omitting the nitrate reductase step. Plasma NO_3^- was calculated as total NO_x minus NO_2^-. The inter- and intra-assay coefficients of variation were 3.4% and 2.7%, respectively, and the lower limit of detection of the assay was 0.24 µM.

In vivo cardiovascular assessment

On P21, one male pup per litter was treated with urethane (1.4-1.5 g kg^{-1} i.p. in water for injections; Sigma, UK) to prevent cardio-respiratory depression and permit controlled manipulation of the cardiovascular system under anaesthesia *in vivo*, as previously described [30-32]. The animal was placed in the supine position on a regulated heating mat and breathed spontaneously on room air. Adequate depth of anaesthesia was continually assessed by the absence of corneal and limb

withdrawal reflexes. When depth of anaesthesia was confirmed, under a dissecting microscope, the left femoral artery was exposed via an incision, individually isolated and instrumented with a catheter pre-filled with heparinised saline (80 i.u. heparin ml^{-1} in 0.9% NaCl). The arterial catheter was connected to a pressure transducer (Argon Division, Maxxim Medical, Athens, Texas, USA). Arterial blood pressure was recorded continually on a custom built Data Acquisition System (Maastricht - Programmable AcQuisition system, M-PAQ and IDEEQ software, Maastricht Instruments, The Netherlands; 1000Hz sample rate). Heart rate was calculated on-line by the programme using the arterial blood pressure pulse to trigger.

Basal blood pressure and heart rate were recorded continuously for at least 10 minutes. Heart rate variability was analysed according to standardised methods (Electrophysiology, 1996). In brief, a 5 minute period of baseline recording was selected and analysed using the heart rate variability function in Labchart 7 (ADI instruments). Normal to normal interval was calculated from the arterial blood pressure trace. In the time domain, the square root of the mean of the sum of the squares of difference between adjacent NN intervals (RMSSD), an established parasympathetic index, was calculated [33]. In addition, the data were fast Fourier transformed to determine frequency specific components. The low frequency (LF), an established sympathetic dominant domain was set between 0.1-1.0 Hz and high frequency (HF), an established parasympathetic dominant domain, was set domain between 1.0-3.5 Hz. The ratio of the LF/HF power was calculated as an index of autonomic balance [33].

In vitro wire myography

Under a bifocal dissecting microscope (Brunel Microscopes Ltd.), the first branch from the femoral artery of the left hind limb (approximate dimensions: internal diameter, 250 µm; external diameter, 440 µm) was excised and placed in ice cold saline solution. The vessel was carefully cleaned of excess connective tissue and cut to a 2 mm long ring. Two 40 µm diameter stainless steel wires were threaded through the lumen of the femoral sections, maintaining the endothelium intact. The wires were then placed between the mounting support jaws of a 4-chamber small-vessel wire myograph (Multi Wire Myograph System 610M; DMT, Aarhus, Denmark) containing warmed oxygenated Kreb's buffer (NaCl 118.5 mM, Fisher, KCl 4.75 mM, Sigma, $MgSO_4.7H_20$ 1.2 mM, Sigma, KH_2PO_4 1.2 mM, Sigma, $NaHCO_3$ 25.0 mM, Sigma, $CaCl_2$ 2.5 mM, Sigma, glucose 11.1 mM, Sigma, UK, gassed with 95% O_2/5% CO_2 mix, 37°C).

Force data from the myograph were recorded at 4 Hz (Labchart 6.0, Powerlab 8/30; AD Instruments, Chalgrove, UK), and each vessel was standardized to an optimal working tension of 100 mmHg as previously described in detail [14]. To test maximal contractile capacity, a potassium (K^+) concentration-response curve was generated following a 20 minute equilibration period by subjecting vessels to increasing doses of a K^+ solution (4.76mM to 100.94mM), washing with Krebs solution twice between doses. Cumulative concentration-response curves to the α_1-adrenergic agonist phenylephrine (PE; 10^{-10}-10^{-5} mol l^{-1}) were determined in half-log increments. The relaxant effects of sodium nitroprusside (SNP; 10^{-10}-10^{-6} mol l^{-1}) and methacholine (MetCh; 10^{-10}-10^{-6} mol l^{-1}) were determined after pre-contraction with phenylephrine (10^{-5} mol l^{-1}).

Langendorff isolated heart perfusion

Rats were killed by CO_2 inhalation and posterior cervical dislocation and the heart was rapidly excised and placed in ice-cold Krebs-Henseleit bicarbonate buffer. It was then cannulated via the aorta (<2 min from excision) and retrogradely perfused through the coronary arteries at 65 mmHg with Krebs-Henseleit bicarbonate buffer solution (120 mmol l^{-1} NaCl; 4.7 mmol l^{-1} KCl; 1.2 mmol l^{-1} $MgSO_2.7H_2O$; 1.2 mmol l^{-1} KH_2PO_4, 25 mmol l^{-1} $NaHCO_3$, 10 mmol l^{-1} glucose, and 1.3 mmol l^{-1} $CaCl_2.2H_2O$; bubbled with 95%:5% O_2:CO_2 mix and warmed to 37°C).

A small flexible non-elastic balloon was inserted into the LV through the left atrium. The balloon was filled with saline and attached to a rigid saline-filled catheter connected to a calibrated pressure transducer (Argon Medical Devices, Texas, USA). The balloon volume was adjusted to get a recording of left ventricular end diastolic pressure (LVEDP) around 5 mmHg. After an initial stabilization period of 15 minutes, basal HR, basal left ventricular systolic pressure (LVSP) and basal left ventricular end diastolic pressure (LVEDP) were recorded. Basal left ventricular developed pressure (LVDP) was calculated as LVSP-LVEDP. The maximum and minimum first derivatives of the left ventricular pressure with respect to time (dP/dt_{max} and dP/dt_{min}) were calculated using an M-PAQ data acquisition system (Maastricht Programmable AcQuisition System, Netherlands). The heart was challenged to a 15 min period of total ischaemia.

Western Blotting

Western blots were performed using 10 µg aliquots of lung protein, resolved on 10% SDS-PAGE gels. Proteins were transferred to polyvinylidene fluoride Immobilon-P membranes (Millipore, UK) by electroblotting. Membranes were blocked at room temperature for 1 h with 5% dry skim milk in Tris-buffered-saline containing 1% Tween-20 (TBS-T; Sigma-Aldrich, UK). Purified antibodies to pro-Surfactant Protein C (1:500 dilution in 5% BSA solution; AB3785, Millipore, UK) were added, and incubated at 4°C overnight. Membranes were washed in TBS-T, incubated for 1 h in a secondary antibody conjugated to horseradish peroxidase (1:1000 dilution in TBS-T, Goat Anti-Rabbit IgG, HRP conjugate (Millipore)) and then washed in TBS-T. Proteins were visualized using enhanced chemiluminescence (Amersham, UK), exposed to X-ray film and films were developed (Fuji FPM100A Processor). Bands densities were quantified and expressed relative to Ponceau S (Image J software, NIH) [34].

Statistical analysis

All values are expressed as mean ± S.E.M. unless stated. Biometry was analysed by the mixed linear model analysis technique with *post hoc* LSD (PASW v18, SPSS inc) to account for multiple observations within litters. All other data were assessed as appropriate using either one-way ANOVA comparing between groups or two-way ANOVA with repeated measures comparing the effects of group, dose or time, in conjunction with the Student-Newman-Keuls *post-hoc* test (Sigma-Stat 3.5; Chicago, IL, USA). For all comparisons, statistical significance was accepted when $P<0.05$.

Results

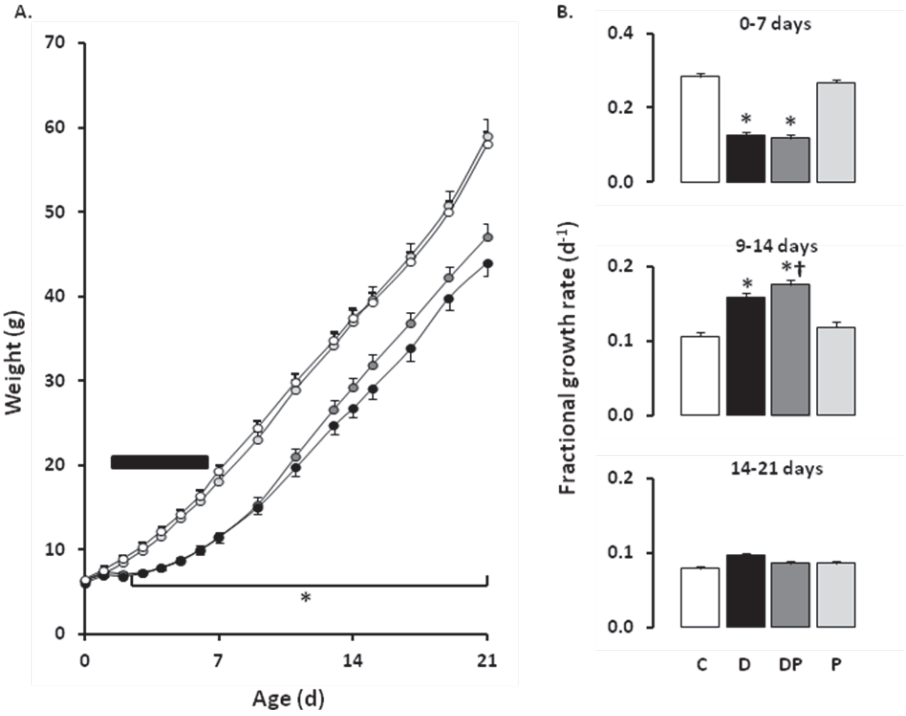
Biometry

Pups were all born spontaneously on day 22 of gestation. There was no significant difference in birth weight between the groups (C, 6.54±0.18 g; D, 6.12±0.16 g; DP, 6.42±0.13 g; P, 6.42±0.15 g). Dexamethasone decreased body weight gain, an effect that was already evident 24 h following the beginning of treatment and continued until P21 (Figure 2A). This effect of dexamethasone on body weight gain was not altered by the addition of pravastatin. Pravastatin alone had no effect on body weight gain relative to control pups. Fractional growth rate (FGR) was calculated for individual animals during the first week of life (P0–7), during the second week life (P9–P14) and the last week before weaning (P14-P21). In control pups, FGR was highest during P0–7 and subsequently decreased during P9–14 and P14–21 ($P<0.05$, Figure 2B). Conversely, in dexamethasone treated pups, with or without pravastatin, FGR was the highest during P9–14 ($P<0.05$, Figure 2B). In pups that received only pravastatin, the FGR pattern was similar to that seen in control pups. Relative to controls, FGR in the dexamethasone treated pups was markedly reduced from P0 to P7 and increased from P9 to P14. A similar trend was observed with the co-administration of pravastatin ($P<0.05$, Figure 2B). However, compared to dexamethasone treated animals, additional administration of pravastatin led to a further increase in FGR between P9-P14. Compared to control animals, dexamethasone treated animals had lighter hearts, livers and kidneys, but these effects were in proportion to their lighter body weight at P21 (Table 1). However, dexamethasone treated pups had relatively larger brains and lungs when expressed as percentage body weight. Concomitant treatment of dexamethasone with pravastatin improved heart and kidney weight compared to dexamethasone treated animals and it reduced the relative increase in lung weight. Compared to control animals, dexamethasone treated pups had significantly shorter crown rump, foot and head lengths, but a significant increase in their ponderal index and in the ratio of brain:liver weight, head diameter:body length and CRL:foot length (Table 2). Concomitant treatment of dexamethasone pups with pravastatin did not have any effect on any of these variables. Pravastatin treatment alone did not affect biometry.

Plasma NO_x

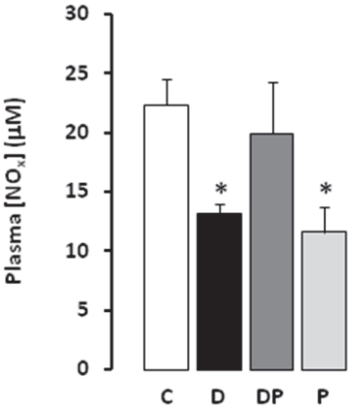
At P21, circulating plasma levels of NO_x were 22.3±2.2 μM in control pups and were similar to values previously reported in rodents [35]. Postnatal treatment with dexamethasone led to a significant decrease in plasma NO_x at P21, whilst this was prevented with concomitant postnatal pravastatin treatment (Figure 3). Postnatal pravastatin treatment alone also significantly decreased plasma NO_x levels compared to control pups at P21.

Figure 2: Postnatal growth curves and growth rates.



Values are mean ± S.E.M. for (A) body weight and (B) fractional growth rate in pups from Control (○, C, n=12 litters), Dex (●, D, n=12 litters), Dex/Pravastatin (◉, DP, n=12 litters) and Pravastatin (⊙, P, n=12 litters) treatment groups. Black bar signifies the six day treatment period. (A) Two way RM ANOVA with post hoc Student-Newman-Keuls. Significant differences (P<0.05): *, C *vs.* D and DP. (B) Mixed liner model analysis with *post hoc* LSD (SPSS); Significant differences (P<0.05): *, *vs.* C; †, D *vs*. DP.

Figure 3: Plasma NO_x concentrations.



Values represent the mean ± SEM for plasma concentrations of nitrate and nitrite levels in Control (□, n=8), Dex (■, n=6), Dex/Pravastatin (■, n=7) and Pravastatin (⧈, n=8) treated animals at postnatal day 21 (P21). Significant differences (P<0.05): *, *vs*. Control. One-way ANOVA with *post hoc* Student-Newman-Keuls test

Table 1: Pup somatic and organ weights at postnatal day 21.

Organ		Control		Dex		Dex / Pravastatin		Pravastatin	
Bodyweight		58.3 ± 1.3		44.0 ± 1.3 *		47.5 ± 1.2 *		59.3 ± 1.4	
Heart	**Abs**	0.310 ± 0.009 †		0.231 ± 0.009 *		0.278 ± 0.011 *†		0.310 ± 0.010 †	
	%	0.525 ± 0.018		0.547 ± 0.019		0.610 ± 0.022 *		0.525 ± 0.020	
Right ventricle	**Abs**	0.055 ± 0.003		0.047 ± 0.003		0.050 ± 0.003		0.061 ± 0.003 †	
	%	0.099 ± 0.004		0.105 ± 0.004		0.106 ± 0.006		0.102 ± 0.004	
Left ventricle + septum	**Abs**	0.209 ± 0.005 †		0.162 ± 0.005 *		0.173 ± 0.006 *		0.220 ± 0.005 †	
	%	0.360 ± 0.012		0.378 ± 0.011		0.372 ± 0.014		0.369 ± 0.011	
Brain	**Abs**	1.35 ± 0.02		1.27 ± 0.02		1.31 ± 0.02		1.33 ± 0.02	
	%	2.35 ± 0.07 †		2.96 ± 0.08 *		2.80 ± 0.08 *		2.20 ± 0.08 †	
Lungs	**Abs**	0.65 ± 0.02		0.59 ± 0.02		0.60 ± 0.02		0.66 ± 0.02	
	%	1.13 ± 0.05 †		1.39 ± 0.05 *		1.27 ± 0.05		1.13 ± 0.06 †	
Liver	**Abs**	2.52 ± 0.08 †		1.90 ± 0.08 *		1.99 ± 0.08 *		2.46 ± 0.09 †	
	%	4.29 ± 0.11		4.33 ± 0.11		4.25 ± 0.12		4.20 ± 0.13	
Kidney	**Abs**	0.344 ± 0.010 †		0.231 ± 0.010 *		0.270 ± 0.010 *†		0.335 ± 0.011 †	
	%	0.598 ± 0.017		0.547 ± 0.017		0.570 ± 0.018		0.571 ± 0.019	
Adrennal	**Abs**	0.013 ± 0.001 †		0.009 ± 0.001 *		0.009 ± 0.001 *		0.011 ± 0.001	
	%	0.022 ± 0.001		0.022 ± 0.001		0.019 ± 0.001		0.019 ± 0.001	

Values are the mean ± SEM for each organ and the % bodyweight for P21 pups from Control (C, n=12 litters), Dexamethasone (D, n=12 litters), Dexamethasone + Pravastatin (DP, n=12 litters) and Pravastatin (P, n=12 litters) treated male pups. Mixed liner model analysis (SPSS). Significant differences (P<0.05): *, *vs.* C; †, D *vs*. DP

Table 2: Pup biometry at postnatal day 21.

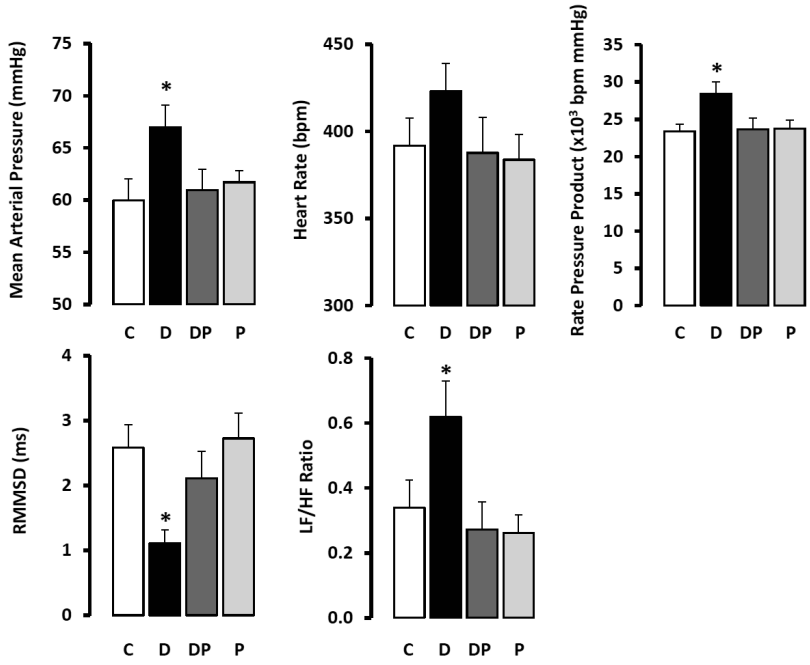
Measure	Control	Dex	Dex/Pravastatin	Pravastatin
Crown Rump Length (mm)	91.3 ± 1.1	78.7 ± 1.1 *	81.7 ± 1.3 *	92.0 ± 1.1
Left Foot Length (mm)	29.5 ± 0.3	27.0 ± 0.3 *	27.7 ± 0.3 *	29.8 ± 0.3
Head Diameter (mm)	15.4 ± 0.2	15.2 ± 0.2	15.7 ± 0.2	15.5 ± 0.2
Head Length (mm)	38.4 ± 0.3	35.4 ± 0.3 *	35.4 ± 0.3 *	37.8 ± 0.3
BMI (kg m^{-2})	7.4 ± 0.0	7.4 ± 0.1	7.8 ± 0.2	7.3 ± 0.1
Ponderal Index (kg m^{-3})	76.0 ± 2.0	90.0 ± 2.0 *	89.0 ± 4.0 *	74.0 ± 2.0
Brain:Liver Ratio	0.54 ± 0.03	0.70 ± 0.03 *	0.66 ± 0.03 *	0.53 ± 0.03
Head Diameter:Body Weight Ratio	0.27 ± 0.01	0.34 ± 0.01 *	0.33 ± 0.01 *	0.26 ± 0.01
CRL: Left foot length	3.1 ± 0.04	2.9 ± 0.04 *	2.94 ± 0.04 *	3.1 ± 0.04

Values are the mean ± SEM for each variable for P21 pups from Control (C, n=12 litters), Dexamethasone (D, n=12 litters), Dexamethasone + Pravastatin (DP, n=12 litters) and Pravastatin (P, n=12 litters) treated male pups. Mixed liner model analysis (SPSS). Significant differences (P<0.05): *, *vs.* C.

Basal cardiovascular function

At P21, in control animals, values for basal arterial blood pressure and heart rate were 60±2 mmHg and 392±16 bpm, respectively, and were similar to previously published measurements in age matched Wistar rat pups under urethane anaesthesia [30]. Postnatal treatment with dexamethasone led to a significant increase in arterial pressure and the rate-pressure product (Figure 4). Concomitant treatment with pravastatin in pups receiving dexamethasone restored arterial basal blood pressure and the rate-pressure product to control levels. Pravastatin treatment alone had no effect on basal cardiovascular variables. At P21, in the time domain, dexamethasone treated pups displayed a lower RMSSD, an indicator of parasympathetic tone (Figure 4). In the frequency domain, dexamethasone led to an increase in the LF/HF ratio, an indicator of sympathetic to parasympathetic balance (Figure 4). Concomitant treatment with pravastatin in dexamethasone treated pups prevented changes in both the time and frequency domains. Pravastatin treatment alone had no effect on heart rate variability.

Figure 4: Basal cardiovascular function *in vivo.*



Values are mean ± S.E.M. for basal mean arterial pressure, heart rate, rate pressure product and for heart rate variability in the time domain (RMMSD) and frequency domain (LF/HF ratio) in P21 pups from Control (□, n=8), Dex (■, n=9), Dex + Pravastatin (■, n=9) and Pravastatin (▨, n=9) treatment groups. Significant differences (P<0.05): *, *vs.* control. One-way ANOVA with *post hoc* Student Newman–Keuls test.

In vitro wire myography

Pups treated with dexamethasone generated lower maximal tension in response to KCl, although there were no changes in sensitivity at P21 (Figure 5A). Concomitant treatment with pravastatin restored KCl-induced contractile responses to control levels. Pravastatin treatment alone had no significant effect.

In contrast, dexamethasone treatment increased the sensitivity and maximal response to PE in isolated femoral arteries (Figure 5B). Concomitant pravastatin treatment prevented the increases in both sensitivity and maximal response to PE. Pravastatin treatment alone had no significant effect on PE induced contractions at P21.

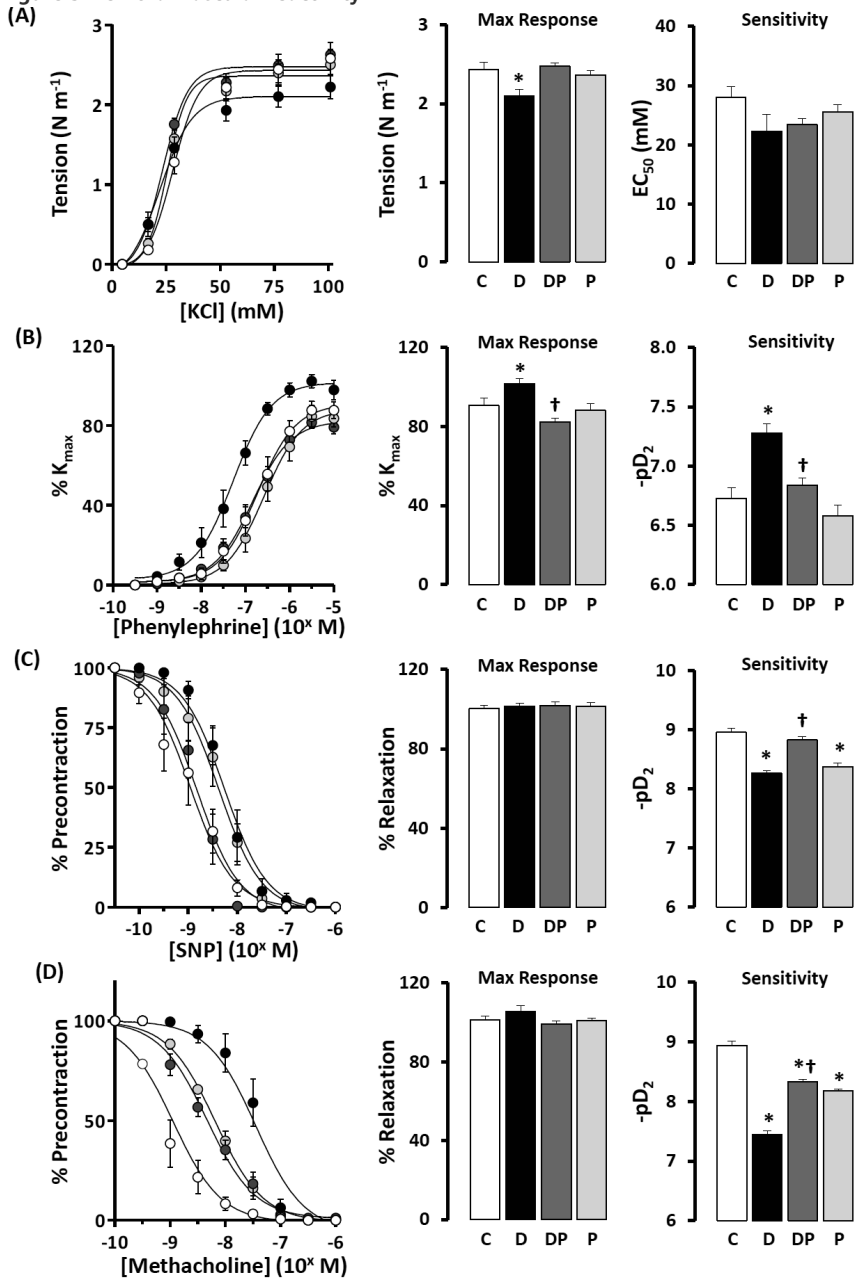
At P21, the sensitivity of endothelial-independent relaxation with SNP of femoral arteries pre-contracted with PE was impaired in dexamethasone treated pups. Concomitant pravastatin treatment in dexamethasone pups restored the dilator function to control levels. Pravastatin treatment alone also caused a decrease in sensitivity to SNP (Figure 5C). Endothelial-dependent relaxation assessed by application of methacholine revealed the same trend of impaired relaxation in dexamethasone treated animals and recovery with concomitant pravastatin treatment (Figure 5D). Pravastatin treatment alone led to a significant fall in endothelial-dependent relaxation sensitivity.

Isolated heart perfusion

At P21, isolated hearts from dexamethasone treated pups displayed lower basal LVDP, coronary flow rates, dP/dt_{max} and dP/dt_{min} when compared to control pups (Figure 6). Concomitant treatment with pravastatin restored all these variables to control values.

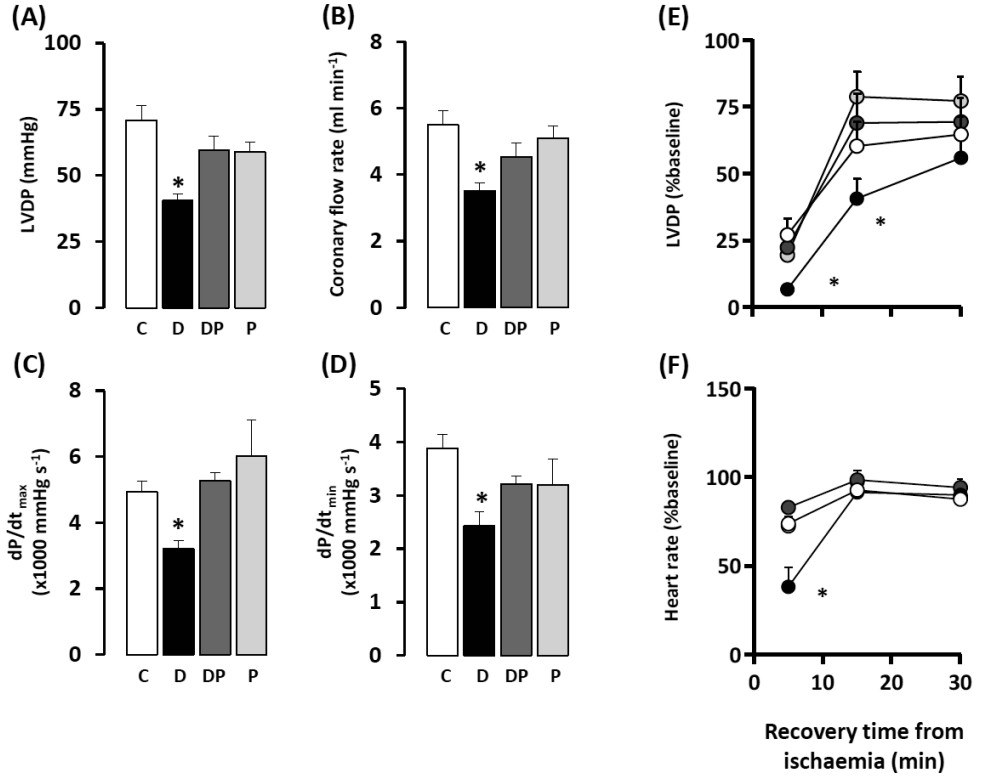
Following ischaemia, hearts from dexamethasone treated animals displayed lower LVDP and heart rates in comparison to control treated animals in the first 10 minutes of recovery, but had comparable values to control animals by 30 minutes. Again, concomitant pravastatin protected against the dexamethasone induced lower LVDP and heart rate following ischaemia.

Figure 5: Femoral vascular reactivity.



Values are the mean ± SEM for the dose-response curve, maximal response and sensitivity to (A), KCl, (B), phenylephrine, (C), SNP and (D), methacholine in femoral arteries from Control (□, n=8), Dex (■, n=7), Dex/ Pravastatin (■, n=7) and Pravastatin (▨, n=7) treated animals. Significant differences (P<0.05): *, *vs* Control; †, Dex + Pravastatin *vs.* Dex. One-way ANOVA

Figure 6: Isolated cardiac function.

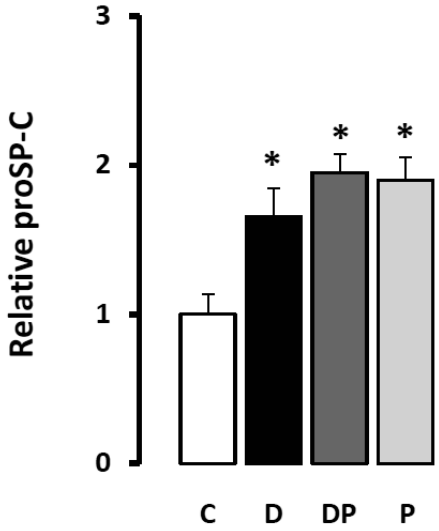


Values are the mean ± SEM for (A), left ventricular developed pressure, (B), coronary flow rate, (C), dP/dtmax, (D), dP/dtmin, (E), left ventricular developed pressure following 15 min ischaemia and (F) heart rate following 15 min ischaemia in Langendorff preparations from Control (□, n=7), Dex (■, n=7), Dex/Pravastatin (■, n=7) and Pravastatin (▣, n=7) treated animals. Significant differences (P<0.05): *, *vs* Control; †, Dex + Pravastatin *vs.* Dex. One-way ANOVA or Two-way RM ANOVA with *post hoc* Tukey test.

Lung molecular biology

Relative to control pups at P21, pups treated with dexamethasone and/or pravastatin showed increased expression of pro-surfactant protein C (Figure 7).

Figure 7: Pulmonary pro SP-C protein expression.



Values represent the mean ± SEM for the expression of proSP-C in pulmonary homogenates quantified by Western Blot Control (□, n=6), Dex (■, n=8), Dex/Pravastatin (■, n=7) and Pravastatin (▨, n=7) treated animals. Significant differences (P<0.05): *, *vs*. Control. One-way ANOVA with *post hoc* Student-Newman-Keuls test

Discussion

It is widely accepted that pre- and postnatal glucocorticoid therapy in the premature neonate can improve respiratory function, yet lead to adverse effects such as stunted growth, endothelial dysfunction and hypertension [3,5,6,12,14,36-38]. Therefore, the identification of the mechanisms underlying the adverse effects of glucocorticoid therapy in premature infants is fundamental to the development of more effective therapeutic options. The postnatal rat is an established experimental model of human prematurity, as postnatal development of cardiovascular, respiratory and neuronal function in this species compares with pre-natal milestones in the human [39-41]. In contrast to experimental animal models involving prenatal treatment with steroids, postnatal glucocorticoid treatment also permits the effects on the offspring to be determined independent of any additional effects on the mother or on the placenta. Using a human clinically-relevant tapering course of postnatal dexamethasone [6,12], this study is the first to test the hypothesis that the detrimental effects of postnatal glucocorticoids on postnatal growth and cardiovascular function are secondary to depleted NO bioavailability, and that these adverse effects will be ameliorated by concomitant postnatal pravastatin. The data show that postnatal dexamethasone decreased NO bioavailability, reduced somatic growth, impaired peripheral vascular function, increased arterial blood pressure and promoted changes in the autonomic nervous system. Further, concomitant treatment with pravastatin in dexamethasone-treated pups alleviated the cardiovascular adverse effects of dexamethasone, enhancing pup growth rate and maintaining

the induction of pulmonary surfactant; changes which were associated with a restoration of plasma NO_x levels to control values. Therefore, the data support the hypothesis tested and suggest that concomitant pravastatin and dexamethasone therapy may maintain the beneficial, while limiting the detrimental, effects of steroids in the perinatal period.

Glucocorticoids, oxidative stress and statins

Accumulating evidence supports that glucocorticoids can deplete NO bioavailability through increases in the production of ROS [42,43]. For example, dexamethasone promotes transcription and activation of the pro-oxidant enzyme xanthine oxidase [18,19,44,45] and dexamethasone can promote ROS generation by the electron transport chain [18] and by NADPH oxidase [18]. Further, glucocorticoids have been shown to decrease the expression of key antioxidant enzymes such as catalase and superoxide dismutase in the rat heart [46], although, importantly, glucocorticoids do induce the expression of other antioxidant enzymes, such as Cu-Zn SOD and glutathione peroxidase in the fetal lung, aiding maturation towards term [47,48]. Increased rate of production combined with decreased removal of ROS by dexamethasone leads to oxidative stress whereby ROS can damage cellular components, particularly in the more fragile newborn period [49]. Glucocorticoids can also affect eNOS expression and function through decreases in eNOS mRNA stability [50] or by limiting the availability of tetrahydrobiopterin (BH_4), a key co-factor for eNOS function [51]. We have previously reported that treatment of newborn rat pups using the same dexamethasone dosing regimen as in the present paper led to significant increases in lipid peroxidation (increased 4-hydroxynonenal content) and significant decreases in the protein expression of heat-shock protein 90, and eNOS in hearts at P21; changes consistent with dexamethasone-induced cardiac oxidative stress with NO depletion [16]. Extending observations in this and other reports [35,50], administration of dexamethasone to postnatal rat pups in the present study also led to significant decreases in plasma levels of nitrates and nitrites (NO_x) at P21, well established indices of circulating NO bioavailability. In the present study, pravastatin was used as a tool to increase NO bioavailability. Accordingly, plasma NO_x levels at P21 were increased to control levels when pravastatin was given in conjunction with dexamethasone in the postnatal period. There are several candidate mechanisms via which pravastatin may lead to sustained increases in NO bioavailability. First, the lipid lowering capacity of pravastatin could increase NO levels as lowering of low density lipoprotein can increase NO bioavailability directly [52]. Second, statins prevent the generation of pyrophosphorylated intermediates of the mevalonate pathway, such as geranylpyrophosphate and farnesylpyrophosphate, through inhibition of HMG-CoA reductase [53]. These intermediates act as important co-factors for intracellular GTPases such as Ras, Rac and Rho [53]. Such GTPases have negative actions on NO bioavailability through promoting eNOS mRNA degradation [26,54] and breaking down Akt, a key activator of NOS [54]. Third, statins may reduce the availability of caveolin-1, a key inhibitor of NO synthesis [55]. Fourth, statins have also been show to have antioxidant abilities via decreasing NADPH oxidase-generated ROS [53].

Hypertension and vascular function

It is well known that perinatal exposure to inappropriate levels of glucocorticoids can increase arterial blood pressure in the fetal and newborn periods [5,6,56-58] and lead to hypertension in later life [12,36,59-63]. In the present study, postnatal dexamethasone treatment led to significant elevations in basal arterial blood pressure at P21. Lack of alterations in basal heart rate at P21 in this study and of cardiac output at 4 weeks of age reported in other studies using a similar model [10], favour an effect of postnatal dexamethasone on peripheral vascular resistance as the main mechanism contributing to hypertension. Whilst established mechanisms mediating glucocorticoid-mediated increases in peripheral vascular resistance may be through increased sympathetic drive [64-66], enhanced responsiveness of the peripheral circulation to α_1-adrenergic agonists [65,67], endothelin[68-70] and angiotensin II [71,72] or impaired endothelial NO synthesis [50,73], only comparatively recently it has become appreciated that the vascular *oxidant milieu* may also profoundly influence peripheral vascular resistance [21,74]. Vascular cells generate ROS, such as the superoxide anion ($^{\bullet}O_2^-$) [75]. Superoxide readily combines with NO, limiting its bioavailability [76]. Hence, under physiological conditions, an increase in the vascular $^{\bullet}O_2^-$:NO ratio will promote vasoconstriction, and the reverse, vasodilatation. In the present study, postnatal dexamethasone administration led to enhanced femoral constrictor reactivity to the α_1-adrenergic agonist phenylephrine, and depressed femoral dilator responsiveness to the endothelium-independent and dependent agonists sodium nitroprusside and methacholine, respectively, at P21. Further data reported reveal that concomitant treatment with pravastatin in dexamethasone-treated pups restored to control values circulating plasma NO_x, the peripheral vasculature constrictor reactivity to phenylephrine and dilator reactivity at P21. Combined, therefore, the data support the concept that glucocorticoids affect vascular function and arterial blood pressure by decreasing NO bioavailability in the peripheral vasculature, which normally acts to oppose constrictor agonists and mediate dilator responses, and that statins restore NO bioavailability normalising the vascular oxidant tone. Interestingly, atorvastatin during pregnancy restored endothelial function in adult rats exposed to a protein restricted diet during pregnancy [77]. This suggests that depletion in NO bioavailability may represent a common mechanism underlying the programming of vascular dysfunction by glucocorticoids or maternal undernutrition and that statins provide a potential therapeutic intervention under both circumstances.

Interestingly, postnatal pravastatin treatment alone caused both endothelial-dependent and -independent vasodilator dysfunction in femoral arteries at P21, and was associated with a decrease in plasma NO_x levels. Whilst an increase in oxidant tone is widely accepted to be detrimental, chronic reductions in ROS generation may also have long-lasting negative effects as free radicals are involved in physiological signalling mechanisms [78]. Through inducing increases in NO bioavailability during the treatment period, pravastatin may down-regulate elements of control of the vascular smooth muscle contractility. Indeed, chronic increases in NO have been reported to down-regulate soluble guanylyl cyclase activity [79,80], to increase phosphodiesterase function [81] and even promote endothelial superoxide generation to restore the vascular oxidant equilibrium between ROS and NO [82]. Such mechanisms could explain the reduced femoral vascular smooth muscle dilator capacity and plasma NO_x levels at P21 following pravastatin

treatment alone in the current study. This reduced vasodilator response may reflect some degree of 'nitrate tolerance', often seen with the chronic usage of NO donors in clinical practice [83]. The results are consistent with previous work from our laboratory where antenatal and postnatal vitamin C treatment alone also leads to impaired NO dependent femoral vasodilator function in later life [14]. In addition, pravastatin may up-regulate other NO lowering systems directly such as the enzyme haemoxygenase-1 (HO-1) [84]. HO-1 degrades cytotoxic haem groups to biliverdin, and in the process produces large amounts of free Fe^{2+} which drives the pro-oxidant Fenton reaction system, therefore depleting NO bioavailability and stimulating oxidative stress [85-87].
The reduced constrictor response in dexamethasone-treated pups to increasing KCl may result from reduced muscle mass and/or alterations in the excitation-contraction-coupling pathway. Studies have shown that glucocorticoids inhibit growth and promote differentiation of vascular tissues *in vitro* implying that decreases in smooth muscle mass may well play a part [88]. Concomitant pravastatin administration in dexamethasone-treated pups was able to prevent this effect, increasing KCl-induced contraction in comparison to pups treated with dexamethasone alone, suggesting that depletion of NO leads to the observed effects. The action of NO on vascular growth is plausible given that NO has pro-angiogenic effects through increasing VEGF via HIF-1 dependent pathways [89,90].
Interestingly, the rate-pressure product was raised in dexamethasone-treated pups indicative of a higher myocardial workload in comparison to control animals [91]. This is likely the result of increased afterload leading to the measured hypertension and shifts in the peripheral vasculature towards a constrictor phenotype. Concomitant postnatal pravastatin in dexamethasone-treated pups may therefore restore the rate-pressure product at P21 by increasing NO bioavailability and normalising peripheral vascular reactivity; effects which have been described in this study.

Despite the hypertension observed *in vivo* and increase in constrictor vascular reactivity *in vitro*, dexamethasone treated hearts displayed impaired pressure generating contractile ability *in vitro*, associated with impaired systolic and diastolic function (dP/dt_{max} and dP/dt_{min}), associated with reductions in coronary flow rate. One interpretation for these observations is that an increase in peripheral vascular resistance may be a compensatory mechanism in the light of a failing heart. Alternatively there may be cardiac failure secondary to an increase in cardiac afterload. Indeed, this model of dexamethasone administration is known to decrease left ventricular thickness [16] and impair systolic contraction with increased end-diastolic volume *in vivo* [10].

Autonomic function

The development of the autonomic control of the cardiovascular system around birth and into postnatal life is dependent on the pre-partum surge in glucocorticoids as fetal adrenalectomy prevents, whilst glucocorticoid replacement restores, increases in blood pressure, renal sympathetic nerve activity and baroreflex sensitivity at birth in sheep [92,93]. In animal models and in humans, both acute and chronic alterations in autonomic control following glucocorticoid administration have been reported. Clinically, glucocorticoids decrease heart rate variability (HRV) both *in utero* [94-97] and in postnatal life [98]. Analysis of heart rate variability in the present study revealed that neonatal dexamethasone treatment significantly decreased the RMSSD in the time domain and led

to a significant increase in the LF/HF ratio in the frequency domain. The result compliments other recent work where antenatal betamethasone also led to a decrease in the RMSSD and a decrease in HF variability in 1.8y old lambs [99]. Combined, past and present data support the underlying idea that glucocorticoids programme a shift in autonomic balance from parasympathetic towards sympathetic dominance. Interestingly, other models of developmental programming including maternal obesity [100], maternal undernutrition [101] and reduced uterine artery blood flow [102] also lead to changes consistent with increased sympathetic control of the cardiovascular system. In the present study, concomitant pravastatin treatment to dexamethasone-treated pups restored all markers of heart rate variation to control levels suggesting that alterations in redox state and depletion of NO during development contribute to the development of basal autonomic control of the circulation. Accordingly, partial restoration of heart rate variability has been reported in simvastatin-treated rabbits with experimental heart failure [103,104] and rosuvastatin improved NO-dependent heart rate and blood pressure variability in apolipoprotein $E^{-/-}$ mice mediated through decreases in caveolin-1 [55].

Growth and development of other organ systems

The negative effects on postnatal growth following dexamethasone administration are well known. Following postnatal glucocorticoid treatment, height and more so weight are significantly decreased in school age children [9]. These findings of reduced asymmetric postnatal growth are supported in animal studies [11,12,16]. In the present study, postnatal treatment with dexamethasone led to suppressed weight gain during the first week of life, most likely due to the anti-proliferative and pro-differentiative effects of dexamethasone at this stage in development [105]. The growth restriction induced by dexamethasone was also asymmetric, reflected by an increase in the ponderal index, relative brain weight and the ratio of brain:liver weight and of CRL:foot length. Between 9 and 14 d of age, the fractional growth rate was increased in dexamethasone-treated pups indicating some degree of '*catch-up*'. This is interesting because both asymmetric growth restriction [106] and catch-up growth [107] are independent strong risk factors for the development of cardiovascular and metabolic diseases in later life [38]. In pups treated concomitantly with pravastatin, the dexamethsone-induced suppression of growth rate was ameliorated but body weight was not restored to control levels at P21. This implies that while increasing NO bioavailability may improve growth, for instance via increasing perfusion and the delivery of oxygen and nutrients [108] and/or promoting angiogenesis [89,90], these effects are not significant enough to revert the powerful stunting effects of steroids in the postnatal period.

Modification of perinatal glucocorticoid therapy to remove unwanted side-effects of the treatment, clearly, is only warranted in a clinical setting if the proposed alterations to standard practice do not themselves prevent the beneficial effects of glucocorticoids on lung maturation. Perinatal glucocorticoid therapy reduces inflammation and promotes surfactant mediated increases in functional residual capacity [1,48,109]. Further, glucocorticoids induce alveolar wall thinning, increasing air space volume whilst decreasing tissue volume, they increase lung antioxidant capacity and accelerate lung fluid reabsorption [47,48,110]. Clinical treatment of preterm neonates with glucocorticoids in either the pre- or postnatal period capitalises on all of these effects and

has significantly reduced the morbidity and mortality associated with infant respiratory distress syndrome and the subsequent development of bronchopulmonary dysplasia and subsequent chronic lung disease in premature babies [3,5,6]. In the present study, dexamethasone treatment with or without pravastatin led to significant increases in the expression of surfactant protein C in the lungs of pups at P21. While concomitant statin treatment significantly diminished the adverse consequences of dexamethasone on the cardiovascular system, it did not affect the established effect of the steroid on pulmonary surfactant synthesis [111,112].

Interestingly, in the present study, treatment of pups with pravastatin alone also led to a significant increase in pulmonary surfactant protein C expression. An emerging number of retrospective studies suggest that statins, when used in patients with chronic obstructive pulmonary disease, can enhance respiratory function, reduce exacerbations and lower hospital admission rates through their *pleiotropic* anti-inflammatory and immuno-modulatory effects [113,114]. In a mouse model of chronic airway inflammation, Ahmad and colleagues also reported that in sensitised lung tissue simvastatin was able to increase the expression of eNOS, reduce nitrotyrosine staining and reduce ADMA (asymmetric dimethyl-arginine, an endogenous inhibitor of eNOS); [115,116]. In premature neonates affected with bronchpulmonary dysplasia, there is general agreement that inhaled NO therapy improves lung function and reduces oxygen dependence in moderately, but not severely, affected neonates [117-120]. The mechanism underlying improved respiratory function is not only mediated through reductions in pulmonary vascular resistance, as cultured human type II alveolar cells exposed to NO have an activated soluble guanylyl cyclase pathway and increased surfactant protein C mRNA expression [121]. Therefore, it is possible that additional pleiotropic effects of pravastatin on lung function are mediated through increases in NO bioavailability in pulmonary tissue.

Clinical implications

In clinical practice today, there is considerable debate surrounding the choice and dosing strategies of glucocorticoids in premature infants [5,6,122]. Hydrocortisone may be used preferentially over dexamethasone to treat premature infants in the postnatal period because hydrocortisone is known to have fewer adverse effects than dexamethasone [1]. Conversely, dexamethasone is known to confer greater beneficial effects on respiratory function than hydrocortisone, established by earlier extubation and decreased dependence on O_2 supplementation [5,6,122]. Greater detrimental and beneficial effects of dexamethasone over hydrocortisone are understandable given the 25x relative potency and longer half-life of dexamethasone in comparison to hydrocortisone [123]. In clinical practice, the greater beneficial effects on respiratory function of dexamethasone over hydrocortisone would therefore make it the glucocorticoid of choice, as long as its detrimental effects could be tamed without affecting its maturational effects. Data in the present study show that concomitant treatment of pups in the postnatal period with dexamethasone and statins diminish the unwanted side-effects of the glucocorticoid on the cardiovascular system while maintaining the induction of pulmonary surfactant. Therefore, the data suggest that the use of statin therapy in conjunction with glucocorticoids in premature infants may be safer than administration of glucocorticoids alone.

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Chapter 7

Neonatal glucocorticoid therapy affects growth patterns in early infancy

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Introduction

Chronic lung disease (CLD) is a significant problem in preterm infants carrying a high risk of mortality and long-term morbidity [1]. Glucocorticoids (GC), especially dexamethasone (DEX) and hydrocortisone (HC) in some neonatal centers, are used to prevent and reduce CLD. Short-term benefits of neonatal GC-therapy to enhance pulmonary development are well-established with the use of either drug [2-5]. However, follow-up studies of children treated with DEX in the neonatal period have reported on increased rates of cerebral palsy and long-term motor deficits and neuropsychological impairments [6-11]. In contrast, similar adverse outcomes have not been found in children neonatally exposed to HC when examined at school age and in adolescence [11,12].

Neonatal GC-therapy has also been associated with impairment of somatic growth, both during treatment ('early growth retardation') [13,14] and at follow-up of formerly DEX-treated but not HC-treated newborns at ages ranging between 2 and 17 years [10-12,15-19]. These follow-up studies on growth in (very) preterm GC-treated infants have generally related (single) measurements of body weight, height, and head circumference at study endpoint to normal growth charts for full-term individuals. However, there is increasing awareness that postnatal growth trajectories estimated from multiple measurements differ considerably between (very) preterm and full-term born infants. Bocca-Tjeertes and others recently showed the former to have lower median weight and height from birth through the first four years [20]. Further, a single biometric measure does not reflect the timing and dynamics of the growth trajectory along which it was attained across postnatal life. Variability in growth pattern has, for instance, been demonstrated in a neonatal rat study using a clinically relevant dose of DEX; the pups showed impaired weight gain during treatment followed by accelerated weight gain (catch-up growth) (Chapter 6 this thesis). Results from several clinical studies have indicated that mainly rapid weight gain, but also rapid height gain in infancy is associated with adverse metabolic and cardiovascular outcomes in later life [21-28], but with improved neurodevelopment in infants [29]. It is presently unclear which impact the combined effects of preterm birth and GC-related early growth retardation may have on subsequent growth; whether GC-treated newborns sometime show catch-up growth presumed to underlie long-term health risks; and whether DEX and HC have differential effects on growth which can be anticipated given some differences in pharmacological characteristics between both drugs [30].

The aim of our study, therefore, was to compare growth patterns for weight, height, and head circumference from birth to age four years, between prematurely born children neonatally treated with DEX or HC and a prematurely born untreated group using recent growth charts especially constructed for preterm infants. Special interest was paid to measures indicative of accelerated growth and its timing.

Patients and Methods

Study Population

This observational cohort study investigates growth patterns from birth to the age of four years in very preterm born infants (< 32 weeks' gestation) who had been treated during the neonatal period with GCs to reduce CLD or had not received this therapy. They were admitted between December 1993 and July 1997 to the NICUs of four clinics in the Netherlands: Wilhelmina Children's Hospital at University Medical Centre Utrecht, Utrecht; Leiden University Medical Centre, Leiden; VU University Medical Center, Amsterdam; and Isala Clinics, Zwolle. The study was approved by the research ethics committee of the University Medical Center Utrecht and written informed consent was obtained from all parents or caregivers. The design of the current study was previously described by Karemaker et al., De Vries et al. and Ter Wolbeek et al., [11,12,31-33]. To reduce CLD, HC therapy in a course starting with 5 mg/kg/day tapering off to 1 mg/kg/day over 22 days was used exclusively in one center (Wilhelmina Children's Hospital at University Medical Centre Utrecht, Utrecht). A course of dexamethasone (DEX) for the same purpose was used at the three other NICUs, starting with 0.5 mg/kg/day tapering off to 0.1 mg/kg/day over 21 days. In each center, the course was sometimes extended or shortened depending on the infant's response to therapy. Treatment indication in all instances was the impossibility to wean from the ventilator together with prolonged dependency on extra continuous oxygen based on the initial phase of CLD. Regular meetings were held by neonatologists to discuss treatment strategies to ensure that centers did not deviate from the treatment protocol which was clinically accepted nationwide. The decision to treat was always left at the discretion of the attending neonatologist. HC-treated children did not receive DEX as a rescue therapy, and vice versa. A third study group consisting of very preterm born infants who had not received neonatal GCs comprised the 'untreated' group. Information on pregnancy and neonatal characteristics, including parity, smoking during pregnancy, antenatal corticosteroid therapy, singleton or twin pregnancy, gestational age at delivery, birth weight, and infant sex was collected from the obstetric database at each center. Neonatal characteristics were taken from NICU medical records: the occurrence of mortality and morbidity, starting date of neonatal GC treatment, duration of ventilator dependency, feeding, and Apgar scores. Children born small for gestational age (SGA) were defined as children whose birth weight and/or birth length was at least 2SD below the mean for the infant's GA. Neonatal data for weight, length (crown-heel length) and head circumference was systematically reviewed from patient records kept by each NICU until discharge. Biometric follow-up data was available from visits to the hospital or from records in Preventive Child Health Care centers. During their first 4 years, children in the Netherlands routinely have about 15 well-child check-ups. These include the assessment of weight, height and head circumference (the latter until the large fontanel is closed). All measurements were made by well-trained health professionals using standard procedures. Only children with ≥ 3 serial biometric measurements and with at least one measurement obtained after 12 months of age were included. Exclusion criteria were: birth weight below 2 SDs of the mean and presence of major congenital anomalies, periventricular leukomalacia or periventricular hemorrhage (≥ grade 3) on neonatal cerebral ultrasound [34].

A total of 210 live born infants was eligible distributed over the three study groups as follows: untreated group (n=105), HC-treated group (n=61) and DEX-treated group (n=44).

Data analysis

Growth curves for weight, height (both 0-4 years) and head circumference (0-1.5 years) at ages adjusted for each completed week of gestation at birth (between 25 and 40 weeks) and for boys and girls separately have been recently published for a large Dutch study population [20]. Apart from gestational age at birth and infant sex, these growth charts were unadjusted for other potentially confounding factors. All serially obtained measurements on individuals in the present study were converted to SD scores (SDS) by using the gestation- and sex-adjusted growth reference charts. Graphs of the individual course of raw SDS at 0-4 years were drawn, separately for boys and girls. Individual growth velocity was determined as the increment in weight or size between two successive measurements divided by the intervening time interval. The resulting absolute growth rate, expressed as g/wk or cm/wk, between successive ages was assumed to be that for the mid-point of this time period. Absolute growth rate curves were explored for each subject and maximal (peak) velocity and age at maximal velocity were determined. Secondly, fractional growth rate was calculated for each period as the absolute growth rate divided by starting weight or size, multiplied by 100 (%/wk), considered a measure of catch-up growth [35].

Statistics

Data management and statistical analysis were performed using SPSS for Windows (version 20, IBM/SPSS Inc., Chicago, Ill., USA). Results were summarized with the use of standard descriptive statistics: counts and percentages for categorical variables, and means and standard deviations (SD) for continuous variables. Groups were evaluated for equivalence in patient characteristics and outcome measures using the Chi-square test for categorical measures and one-way ANOVA for continuous variables, followed by post hoc comparisons using the Fisher exact test and Dunnet's correction for multiple comparisons as appropriate. Variables not-normally distributed were transformed (natural log, square root).

The serial measures (SDS; growth velocities) available for each infant were analyzed with linear mixed-effects modeling to produce individual growth trajectories. Mixed-effects models allow for intra-infant correlation of repeated measurements, make use of the exact age at measurement, and account for a dissimilar number of measurements on each infant. Such models also allow for individual variation in growth trajectories, as random effects permit variability in intercept, slope, and curvature between subjects. For each biometric variable, we explored linear, quadratic and cubic functions of age (time at birth; t=0 months). The age terms were included as both fixed and random effects. Neonatal treatment with GCs was included as a main effect and also as an interaction with the age terms. Models for SD-scores were unadjusted for potential co-variables (see above), while gestational age at birth, birth weight, and twin pregnancy were included in the models for growth velocity. With all tests, significance was assumed at the level of $\alpha = 0.05$ (two-sided).

Results

Participant characteristics are summarized in Table 1. The untreated and GC-treated groups did not differ statistically with regard to maternal parity, antenatal GC therapy, infant gender and 5-min Apgar score. Compared to the untreated group, infants in the treated groups were born earlier, weighed less at birth, and were less frequently a twin member (significant for DEX group). However, the group differences in birth weight disappeared after adjustment for gestational age at birth and gender, as evidenced by the birth weight SD scores. In the neonatal period, GC-treated infants had a higher prevalence of IRDS (grades 1-4) and severe IRDS (grades 3-4) and needed more often artificial ventilation and for a longer period of time than untreated infants. The onset of GC-therapy occurred significantly later in the DEX group (mean 11.7, range 3-42 days) than in the HC group (mean 20.7, range 5-72 days).

We analyzed a total of 3245, 2645, and 1397 measurements for weight, height and head circumference, respectively, with an average of 15 (range 3-35), 13 (range 3-26) and 8 (range 3-17) measurements per child for these biometric variables, respectively. The final measurement of both weight and height was made at 41 months (mean; range 15-51), and at 13 months (range 5-18) for head circumference. These numbers and ages did not differ statistically across the study groups or between boys and girls (p-values not shown).

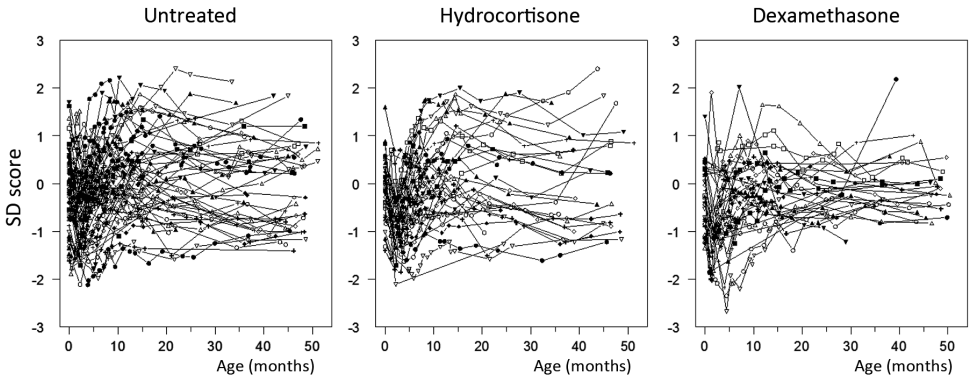
Table 1. Baseline characteristics of the study population.

	Untreated (N=105)	Hydrocortisone (N=61)	Dexamethasone (N=44)	ANOVA or Chi-square; p-value ¶
Pregnancy characteristics				
Nulliparous (n; %)	68 (64.8)	41 (67.2)	26 (59.1)	X^2=0.76; p=.685
Antenatal corticosteroids (n; %)	62 (59.0)	44 (72.1)	26 (59.1)	X^2=3.17; p=.205
Twins (n; %)	52 (49.5)	21 (34.4)	10 (22.7) ‡	X^2=10.25; p<.01
Neonatal characteristics				
Gestational age at birth (wk)	29.1 ± 1.5	28.3 ± 1.5 ‡	27.7 ± 1.8 ‡	F(2,207)=14.54; p<.0001
Birth weight (g)	1187 ± 280	1057 ± 205 ‡	1021 ± 229 ‡	F(2,207)=9.08; p<.0001
Birth weight SD score #	0.01 ± 0.91	-0.21 ± 0.74	-0.30 ± 0.78	F(2,207)=2.24; p=.092
Male gender (n; %)	52 (49.5)	33 (54.1)	30 (68.2)	X^2=4.37; p=.112
5-min Apgar score	8.1 ± 1.8	7.7 ± 1.6	7.6 ± 1.9	F(2,207)=1.70; p=.186
5-min Apgar score < 7 (n; %)	17 (16.2)	12 (19.7)	10 (22.7)	X^2=0.95; p=.623
Breast feeding (n; %)	53 (50.5)	27 (44.3)	13 (29.5)	X^2=5.51; p=.064
Ventilator dependency (n; %)	81 (77.1)	59 (96.7) †	44 (100) ‡	X^2=21.50; p<.0001
Ventilator dependency (days)	11.0 ± 15.3	16.9 ± 9.9 † *	27.3 ± 13.1 ‡	F(2,181)=21.79; p<.0001
Ventilator dependency ≥ 7 days (n; %)	44 (54.3)	53 (89.8) ‡	43 (97.7) ‡	X^2=38.54; p<.0001
Age at start GC therapy (days)	----	11.7 ± 10.0	20.7 ± 9.6	t-test; p<.0001
IRDS all grades (n; %)	57 (54.3)	50 (82.0) ‡	35 (79.5) ‡	X^2=15.06; p<.001
IRDS grade 3-4 (n; %)	23 (40.4)	33 (66.0) ‡	25 (71.4) ‡	X^2=11.07; p<.005

Data are Mean ± SD or number (%). GC therapy: glucocorticoid therapy.
adjusted for gestational age at birth and gender [20].
¶ One-way ANOVA or Chi-square testing for significant differences across the three study groups.
† p<0.05; ‡ p<0.01; post hoc test: hydrocortisone/dexamethasone group *vs.* untreated group (adjusted for multiple comparisons).
* p<0.01; post hoc test: hydrocortisone group *vs.* dexamethasone group (adjusted for multiple comparisons).

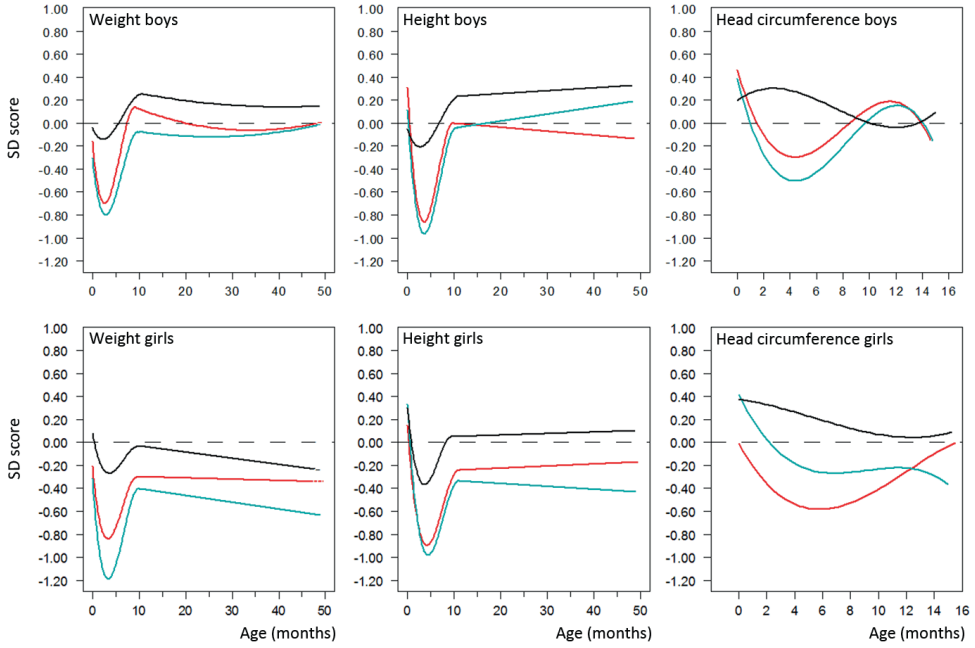
Weights and sizes were converted into SDS which had normal distributions (mean 0, SD 1). Figure 1, as an example, shows the course of body weight SDS for individual boys in each study group from birth to 4 years of age. The GC treated boys showed a transient fall in SDS in early infancy, which was also apparent in the graphs for height and head circumference (both boys and girls). To capture this fall in SDS, mixed-effects models were applied to the serial SDS in two steps, from birth to 12 months and from 12 to 48 months. Data were best fitted by a cubic function of age on step 1 (see Appendix 1 for model estimates) and by a linear or quadratic function of age on step 2 (model estimates not shown). The mean growth trajectories over the first 4 years (weight, height; head circumference till 15 months) are shown in Figure 2 by sex and treatment group. Subsequent to GC therapy, there was a significant fall in SDS for weight and height (about -1 SD) and to a lesser extent for head circumference (about -0.6 SD) at 2-5 months of age. For boys, the growth trajectories of the three groups converged by about one year and showed normal growth thereafter (Figure 2, *a,c,e).* For girls, the growth trajectories did not fully converge and biometry remained reduced over time in the GC-treated groups, the DEX group in particular (Figure 2, b,d,f). However, results from both steps of analysis showed that the model-predicted SD scores both at birth and at 1 and 4 years of age for both sexes did not differ statistically between the three groups.

Figure 1.



The course of body weight SD-scores in individual male children in each study group from birth to 48 months of age.

Figure 2.



Growth in weight of males (a) and females (b), in height of males (c) and females (d) and in head circumference of males (e) and females (f), ages 0 to 4 in DEX (blue line), HC (red line) and untreated (black line) infants using SD-scores.

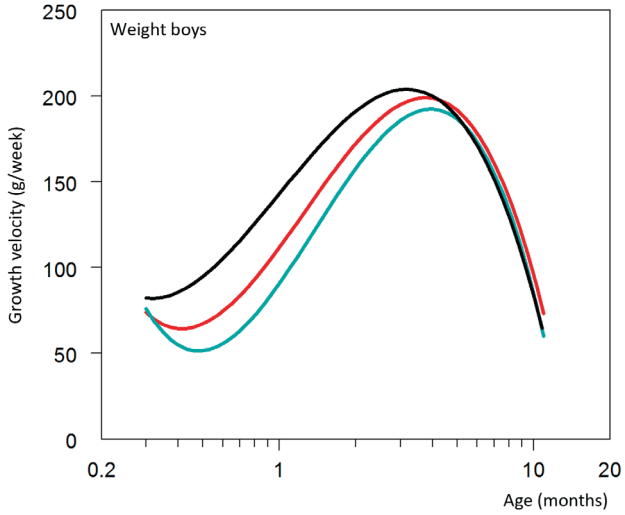
To study infant growth between birth and 1 year in detail, absolute growth velocities for weight and size were subjected to linear mixed-effects modeling (cubic models) and analyzed by sex and treatment group. Age at birth, birth weight and singleton/twin pregnancy were included in the models as potential covariates. Only birth weight appeared of statistical importance, with lower growth velocities in infants born heavier. Graphic presentation of the six resulting models (estimates not shown) is confined to that for male body weight (Figure 3), as all absolute growth rate curves had similar features. GC-treated infants compared to untreated infants showed significantly lower growth velocities for weight and size at 1 month of age. Thereafter, velocities increased in medicated infants and the shape of their curve of velocity magnitude resembled that seen in untreated infants, although with delay in time (Figure 3). This 'shift of curves to the right' in medicated infants was also found for the age at which maximal (peak) growth velocity of weight/size was reached in individual boys and girls, whereas peak velocities, adjusted for birth weight, were generally similar across the study groups, except for weight and head circumference in DEX-treated girls (Table 2).

Table 2. Maximal (peak) growth velocity of infant weight and size and age at achievement.

	Males ¶			Females ¶		
	Untreated	Hydrocortisone	Dexamethasone	Untreated	Hydrocortisone	Dexamethasone
Body weight						
Age at maximal velocity (months)	3.4 (1.7)	4.2 (1.7)	4.7 (1.8) †	2.9 (1.5)	4.4 (1.6) ‡	5.2 (1.9) ‡ *
Maximal velocity (g/wk)	247 (54)	239 (52)	221 (55)	217 (39)	204 (39)	185 (47) †
Body height						
Age at maximal velocity (months)	2.8 (2.1)	3.9 (2.1) †	4.8 (2.1) ‡ *	2.4 (1.4)	3.7 (1.5) ‡	3.9 (1.8) †
Maximal velocity (cm/wk)	1.23 (0.4)	1.15 (0.4)	1.02 (0.4)	1.12 (0.4)	1.13 (0.5)	1.05 (0.5)
Head circumference						
Age at maximal velocity (months)	1.6 (1.2)	2.5 (1.1) ‡	3.0 (1.2) ‡	1.7 (0.8)	2.4 (0.9) ‡	3.2 (1.0) ‡ *
Maximal velocity (cm/wk)	0.82 (0.2)	0.75 (0.2)	0.69 (0.2)	0.81 (0.4)	0.80 (0.4)	1.0 (0.7) †

Data are Mean ± SD, adjusted for birth weight.
¶ Multivariable ANOVA testing for significant differences across the three study groups.
† $p<0.05$; ‡ $p<0.001$; post hoc test: hydrocortisone/dexamethasone group *vs.* untreated group (adjusted for multiple comparisons).
* $p<0.01$; post hoc test: hydrocortisone group *vs.* dexamethasone group (adjusted for multiple comparisons).

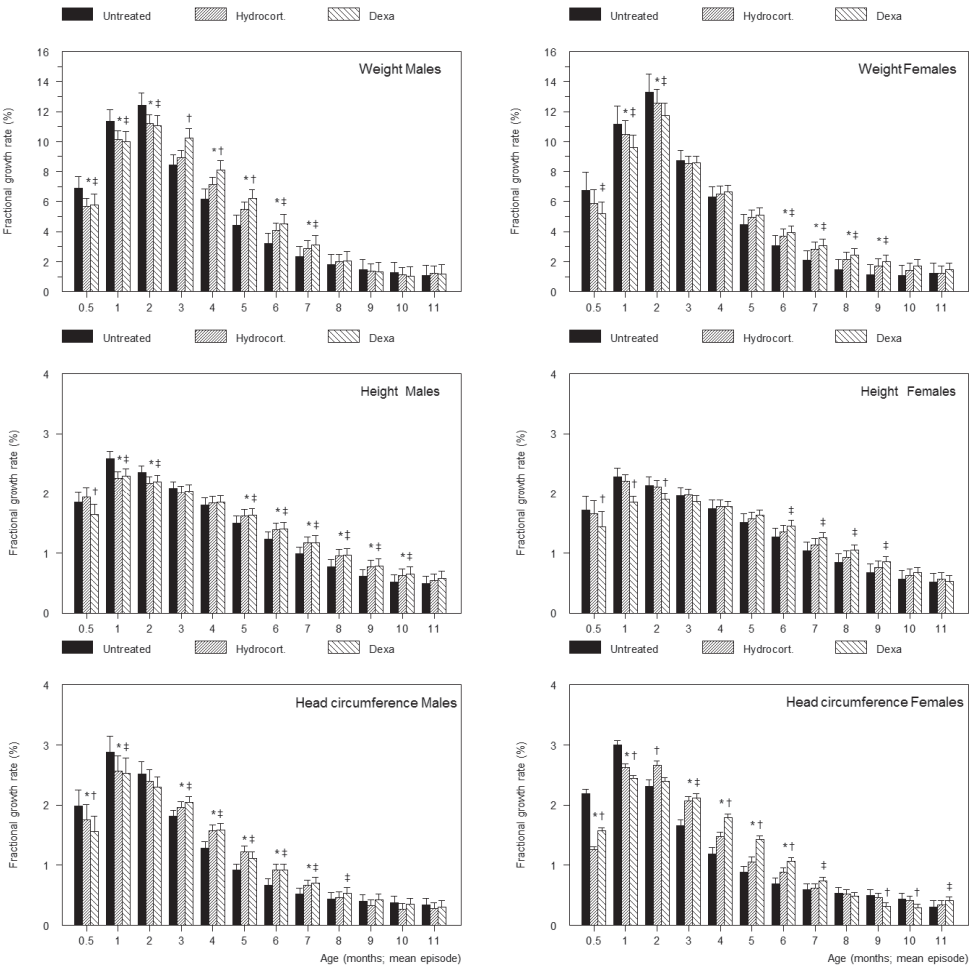
Figure 3.



Growth velocity for weight separate for boys in DEX (blue line), HC (red line) and untreated infants (black line). Note that age is on a logarithmic scale and reflects the midpoint of successive measurements, centered at one month of age.

As a third parameter of growth velocity we studied fractional growth rate (FGR; %week^{-1}) with the use of linear mixed modeling (cubic models). Model estimates are given in Appendix 2 and model-predicted values at 1-month intervals during the first year are presented in Figure 4. Note that age reflects the midpoint of the period for which FGR was determined. In GC-treated infants compared with untreated infants, FGR was generally reduced during the first 2 months, followed by an episode of increased FGR-values for which the timing and duration varied for the three biometric variables and infant sex, but not for the type of GC-treatment. For instance, the onset and end of increased FGR-values for weight was different between boys (3-7 months) and girls (6-9 months) and to some extent also for height (Figure 4).

Figure 4.



Fractional growth rate (%/week) for weight, height, head circumference in DEX, HC and untreated boys and girls. * P<0.05 HC vs. untreated, ‡ P<0.05 DEX vs. untreated, † P<0.05 DEX vs. both HC and untreated. Analyzed with linear mixed-effects modeling.

Discussion

The current findings indicate that neonatal intervention with DEX and HC affected growth patterns for weight, height and head circumference in the first 48 months in preterm born males and females, when comparing their growth to a preterm born not GC-treated reference group. Effects of DEX and HC treatment were observed mainly on growth velocities. Compared to untreated infants, the growth velocity curves of treated neonates showed a shift to the right, representing

a delay in time. They presented during and shortly after treatment decreased absolute growth velocities, with thereafter an increase in growth velocity. A shift to the right was also seen for the age at which maximal growth velocity of weight/size was reached in boys and girls. FGRs of weight, height and head circumference were generally reduced in the treated groups during the first two months of age, with catch up growth in the following months. In DEX-treated infants these changes were more pronounced than in HC-treated infants.

The major strength of this study is primarily its longitudinal approach. Former studies reporting on the effects of neonatal GC-therapy on growth compared time points not growth patterns [10-12, 15-19]. A second strength is the use of reference growth curves specifically developed for preterm born infants and built up from longitudinal data for weight, height, and head circumference. Previously, reference growth charts for preterm infants were often based on cross-sectional data [36-38].

GCs exert their influence through glucocorticoid and mineralocorticoid receptors, which are expressed in all body parts. Child growth is multifactorial and as GCs have many target physiological and biochemical mechanisms, growth and GCs collide at several points. Insulin-like growth factor-1 (IGF-1) plays an important role in both antenatal and postnatal growth [39,40]. Excess of GCs intervene with the growth-hormone-IGF-1-axis (GH-IGF1-axis) at different levels (hypothalamic, pituitary and target organ), having an impact on hormone release, receptor abundance, signal transduction, gene transcription, pre-mRNA splicing and mRNA translation [40]. Differences in impact on FGR and maximal velocity between DEX and HC may be caused by the pharmacological and biological characteristics of the two GCs, such as differences in binding preference to the glucocorticoid receptor (DEX) or mineralocorticoid receptor (HC) [41]. Given that DEX and HC differ in potency, the dose of DEX administered in this study is 2.5 times more potent than the HC dose [42]. Therefore, we cannot conclude that DEX is more harmful than HC. Fewer effects on growth in the HC-treated infants may indicate overtreatment in the DEX-treated group. However, the DEX regimen used in this study was comparable to treatment schedules at other NICUs in that period of time [43].

Immediately after term birth the fastest growth in the entire life span occurs. Moreover, several regulatory mechanisms are not functionally mature at the end of gestation but continue to develop during the postnatal period, especially when infants are born preterm. Therefore, variations in this process may have long lasting effects on health. It is yet not clear what the most healthy, optimal growth pattern is and how it can be achieved. Rapid weight gain in infancy, mainly after a period of growth restriction ('catch-up' growth) is an important risk factor for subsequent obesity [44,45], coronary heart disease [46], hypertension [21,47] and insulin resistance [48]. Furthermore, height growth velocity is reported to be significantly positively associated with blood pressure and waist circumference in adulthood [49]. The period of increased weight and height FGR in the DEX and HC treated infants in this study may therefore have important consequences for the development and the expression of disease later in life. A mechanism by which accelerated weight gain make increased susceptible to cardiovascular and metabolic diseases in adulthood is through alterations in body composition, including a disproportionately high fat mass, associated with a lower metabolic rate and insulin resistance. It has been suggested in full terms of diabetic

mothers that altered hormonal levels present in the immediate postnatal period result in lasting malprogramming of neuroendocrine systems (particularly in the hypothalamus) crucially involved in the regulation of appetite, body weight homeostasis, and metabolism [50]. In contrast, rapid growth (weight and height) in infancy is positively associated with the development of cognition and mental health at school age [29], whereas mental illness [51] is among one of the adverse health consequences associated with slower growth. However, both studies were conducted in full term infants. A potential biological mechanism underlying the positive association observed in the study of Yang is the GH-IGF-1 axis [29]. Van Pareren et al., have shown that children born small for gestational age who underwent GH treatment showed not only catch up growth in height but also improvements in IQ and problem behavior scores [52].

As regards to head circumference; a close relation has been reported between head circumference growth, total brain volume [53] and neurocognitive development (IQ, school performance) of premature infants in childhood [54,55]. Head circumference catch-up growth during the first postnatal year of preterm born infants, was positively correlated with neurodevelopmental outcome later in life [27]. In a retrospective cohort study of preterm born neonates, head circumference growth between birth and three months corrected age was significantly higher in those with unimpaired neuromotor and Bayley scores than in those with mild or severe impairment [27]. So the critical period for the influence of factors on head circumference catch-up and neurodevelopment is between birth and three months corrected age. This is exactly the time window when GC-therapy is started and head circumference growth is initially delayed. However, all conclusions of the above-mentioned growth studies are made independent of GC-treatment. If DEX and HC increase the risk of long term health consequences, by increasing poor growth during the first month and increased growth velocity thereafter, would be an interesting subject of further study.

We also recognize some limitations; we used small groups, especially the group of DEX females was small. A lot more children were included in the original NEOCORT database [11,12,31-33]. However, to compare growth patterns we used strict inclusion criteria, e.g. that individuals needed to have at least 3 serial biometric measurements to be included in the analysis. Furthermore, we did not have information about factors that are known to influence neonatal growth, including parental height, feeding problems, infections and other neonatal complications [56-58]. Finally, our study had a retrospective design. A major problem of these studies is to find a comparable untreated group because infants treated with GCs are often born more prematurely, at a lower birth weight, and have more serious (pulmonary) morbidity. Ideally, a randomized controlled trial should be undertaken to investigate the effects of neonatal DEX and HC on growth. However, in view of the effects of DEX courses used in the nineties, this is no longer possible since the dose regimen for the use of DEX has changed. Even so, we are confident that our data are reliable, because measurements were done with standardized equipment and techniques, and by professionals who were trained for measuring children. In conclusion, our findings indicate that growth patterns of preterm born infants were affected by GC-treatment, more by DEX than by HC. The findings may have impact on health in later life for those individuals treated with GCs in the neonatal period.

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Appendix 1. Statistical results of linear mixed modelling for growth SD scores between 0 and 12 months.

Variable	Body Weight		Body Height		Head Circumference	
	Boys	Girls	Boys	Girls	Boys	Girls
Intercept	-0.116 (0.108)	0.116 (0.113)	-0.084 (0.144)	0.285 (0.142) *	0.205 (0.127)	0.375 (0.140) †
Main Effects						
Treatment						
Hydrocortisone (Group 2)	-0.065 (0.173)	-0.332 (0.192)	0.431 (0.253)	0.019 (0.233)	0.244 (0.205)	-0.398 (0.236)
Dexamethasone (Group 3)	-0.219 (0.179)	-0.425 (0.241)	0.196 (0.347)	0.094 (0.363)	0.216 (0.223)	0.070 (0.301)
Untreated group (Group 1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Time						
Age linear	-0.152 (0.040) ‡	-0.237 (0.041) ‡	-0.216 (0.064) ¶	-0.499 (0.055) ‡	-0.0047 (0.048).	-0.088 (0.047)
Age squared	0.044 (0.0085) ‡	0.054 (0.0074) ‡	0.0513 (0.012) ‡	0.093 (0.012) ‡	-0.0049 (0.008).	0.0087 (0.0081)
Age cubic	-0.0024 (0.0005) ‡	-0.0028 (0.0004) ‡	-0.0027 (0.00069) ‡	-0.0046 (0.0007) ‡	0.00032 (0.0004)	-0.00028 (0.0004)
Interaction Effects						
Group 2 x Age linear	-0.239 (0.065) ‡	-0.184 (0.067) †	-0.483 (0.120) ‡	-0.344 (0.089) ‡	-0.422 (0.079) ‡	-0.256 (0.079) ¶
Group 3 x Age linear	-0.219 (0.073) †	-0.161 (0.088)	-0.363 (0.171) *	-0.352 (0.149) *	-0.399 (0.091) ‡	-0.413 (0.122) ¶
Group 1 x Age linear	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Group 2 x Age squared	0.046 (0.014) ¶	0.039 (0.012) ¶	0.081 (0.021) ¶	0.054 (0.019) †	0.073 (0.014) ‡	0.041 (0.014) †
Group 3 x Age squared	0.047 (0.016) †	0.026 (0.016)	0.056 (0.029)	0.052 (0.029)	0.074 (0.016) ‡	0.070 (0.021) ¶
Group 1 x Age squared	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Group 2 x Age cubic	-0.0023 (0.0008) †	-0.0019 (0.0007) †	-0.0038 (0.0011) ¶	-0.0023 (0.0011) *	-0.0031 (0.0007) ‡	-0.0016 (0.0006) *
Group 3 x Age cubic	-0.0025 (0.0096) *	-0.0009 (0.0009)	-0.0023 (0.0015)	-0.0021 (0.0016)	-0.0034 (0.0008) ‡	-0.0030 (0.001) †
Group 1 x Age cubic	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Data are presented as estimates (SE) of the models.

‡ $p < 0.0001$
¶ $p < 0.001$
† $p < 0.005$
* $p < 0.05$

Appendix 2. Statistical results of linear mixed modelling for fractional growth rate between 0 and 12 months.

Variable	Body Weight		Body Height		Head Circumference	
	Boys	Girls	Boys	Girls	Boys	Girls
Intercept	22.71 (1.05) ‡	22.36 (0.75) ‡	3.34 (0.21) ‡	2.95 (0.24) ‡	5.06 (0.19) ‡	4.77 (0.24) ‡
Main Effects						
Treatment						
Hydrocortisone (Group 2)	-5.01 (1.56) ¶	-2.26 (0.74) †	-0.55 (0.20) †	-0.19 (0.24) n.s.	-1.29 (0.26) ‡	0.82 (0.40) *
Dexamethasone (Group 3)	-2.74 (1.70) n.s.	-2.73 (0.94) †	-0.53 (0.29) *	-0.75 (0.34) *	-0.68 (0.30) *	-1.82 (0.49) ‡
Untreated group (Group 1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Time						
Age linear	-4.95 (0.56) ‡	-4.64 (0.38) ‡	-0.164 (0.092) *	-0.047 (0.109) n.s.	-1.27 (0.098) ‡	-1.302 (0.134) ‡
Age squared	0.46 (0.096) ‡	0.37 (0.065) ‡	-0.029 (0.015) *	-0.036 (0.017) *	0.131 (0.016) ‡	0.151 (0.024) ‡
Age cubic	-0.0146 (0.0048) ¶	-0.009 (0.0034) ‡	0.0023 (0.0007) ¶	0.0023 (0.0008) †	-0.0046 (0.0008) ‡	-0.0060 (0.00120) ‡
Birth weight	-0.0025 (0.00038) ‡	-0.0024 (0.00033) ‡	-0.00044 (0.00008) ‡	-0.00053 (0.0001) †	-0.00039 (0.00007) ‡	-0.00036 (0.00009) ‡
Interaction Effects						
Group 2 x Age linear	2.85 (0.92) ¶	0.73 (0.26) †	0.178 (0.06) †	0.055 (0.081) n.s.	0.780 (0.156) ‡	-0.167 (0.242) n.s.
Group 3 x Age linear	2.46 (1.01) *	0.87 (0.35) *	0.164 (0.09) *	0.230 (0.109) *	0.437 (0.168) †	1.325 (0.309) ‡
Group 1 x Age linear	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Group 2 x Age squared	-0.445 (0.156) †	-0.051 (0.021) *	-0.012 (0.0047) *	-0.0037 (0.006) n.s.	-0.122 (0.026) ‡	0.0037 (0.042) n.s.
Group 3 x Age squared	-0.443 (0.172) *	-0.058 (0.028).*	-0.011 (0.0063) n.s.	-0.0157 (0.0078) *	-0.069 (0.027) *	-0.231 (0.056) ‡
Group 1 x Age squared	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Group 2 x Age cubic	0.0204 (0.0078) *	n.s.	n.s.	n.s.	0.0055 (0.0013) ‡	0.00041 (0.0022) n.s.
Group 3 x Age cubic	0.0216 (0.0086) *	n.s.	n.s.	n.s.	0.0031 (0.0013) *	0.0114 (0.0031) ‡
Group 1 x Age cubic	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Age centred at 1 month. Data are presented as estimates (SE) of the models.

‡ $p < 0.0001$
¶ $p < 0.001$
† $p < 0.005$
* $p < 0.05$

PART III

Chronic fetal hypoxia

Chapter 8

Allopurinol prevents object recognition memory impairment in adult offspring following chronic fetal hypoxia

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This preliminary study will be published alongside a comprehensive evaluation of cerebral structure which is currently in progress

Abstract

Many metabolic and cardiovascular diseases in later life, including type 2 diabetes and hypertension, are developmental in origin and can arise from sub-optimal intra-uterine environments. It is unclear whether chronic fetal hypoxia, as occurs in placental insufficiency, causes a risk for neurological impairment in later life. The aim of this preliminary study was to investigate in rats the effects of chronic fetal hypoxia on behaviour and cognitive function in adult offspring, and to determine whether the xanthine-oxidase inhibitor allopurinol has any neuroprotective effects.

Methods

Pregnant Wistar rats were exposed to normoxic (21% O2; N, n=11) or hypoxic conditions (13% O2; H, n=9), with or without allopurinol (30mg.kg-1.d-1 in jelly; NA, n=9; HA, n=10) from gestational days 6-20. At 3.5 months of age, anxiety-related behaviour and cognitive function were assessed using an elevated plus maze and the object recognition task (OR), respectively.

Results

At 4 months of age, body and brain weights were not different between the groups. Compared to normoxic offspring, object recognition was significantly reduced 3 hours after testing in adult offspring of hypoxic pregnancy. This ratio remained attenuated 24 hours after testing in offspring of hypoxic pregnancy, suggesting long-term memory impairment. These effects were prevented in offspring of hypoxic pregnancy treated with allopurinol. Anxiety-related behaviour was not different between the groups.

Conclusions

Chronic fetal hypoxia can impair cognitive function in adulthood; an effect that can be ameliorated by maternal allopurinol treatment. Xanthine oxidase-mediated oxidative stress may be an important link in the developmental origins of neurological disease.

Introduction

An adverse prenatal environment has been shown to increase the risk of adult disease [1]. Intrauterine stressors that initiate fetal programming include undernitrition, glucocorticoid exposure and placental insufficiency [2,3]. Reduced oxygenation is one of the most common challenges to the fetus during gestation. It can arise from placental insufficiency [4], high altitude environments [5], maternal respiratory disease [6] or maternal smoking [7]. In relation to the brain, placental insufficiency is thought to cause impairments in cognitive function in the postnatal period [8-11] and may be associated with psychopathology in later life (e.g. schizophrenia [12]. As placental insufficiency reduces both oxygen and nutrient supply, it is difficult to establish whether the adverse effects on cerebral function are due to under-oxygenation or under-nutrition [13]. Animal models have been employed to isolate the effects of hypoxia from undernutrition. These include the chick embryo [14-17] and a rat model developed in our laboratory using chronic inhalational hypoxia (13% O2 days 6-20 of gestation), which does not alter maternal food or water intake [18-22].

The mechanism via which developmental hypoxia programmes fetal development and long-term health is not well understood. However, recent studies have suggested that fetal oxidative stress may contribute to permanent alterations in the offspring through programming mechanisms [23,24]. Hypoxia is a potent stimulus for the generation of reactive oxygen species (ROS) [25]. ROS serve as signaling molecules that induce transcription of several genes important in oxygen sensing, cell differentiation, and proliferation [26,27]. However, when the generation of ROS exceeds the capacity of antioxidant defense mechanisms, oxidative stress occurs [28]. ROS can directly interact with proteins and DNA to oxidize amino acids, which disrupt normal structure and function. Additionally the fetus is highly sensitive to injury of oxidant molecules because of its low antioxidant capacity [29,30]. Work in our group has recently shown that fetal hypoxia during most of gestation in the rat is associated with oxidative stress [19,21]. Administration of the xanthine-oxidase inhibitor allopurinol can reduce the production of free radicals [31,32]. Indeed, allopurinol can limit brain damage by reducing the formation of free radicals after acute hypoxia [31,33-35]. Furthermore, allopurinol has a direct free radical (hydroxyl) scavenging effect [36]. We conducted a preliminary functional study in rodents to investigate the effects of chronic fetal hypoxia on behaviour and cognitive function in adult offspring, and determined whether allopurinol had any neuroprotective effects.

Methods

Ethical Approval

The study was approved by the Cambridge University Ethical Review Committee. All procedures were carried out under the UK Animals (Scientific Procedures) Act 1986 and conducted under the authority of the appropriate project license.

Animals and Experimental Design

Wistar rats (Charles River Ltd., Margate, UK) were housed under standard conditions (60% humidity, 23±1°C and a 12:12-hour light-dark cycle) with free access to food (Special Diet Services, UK) and water. After 10 days of acclimatisation, virgin female Wistar rats (n=48, 10-12 weeks of age) were paired individually with male Wistar rats (minimum 12 weeks of age). The presence of a copulatory plug was considered day 0 of pregnancy (term is ca. 22 days). Upon the confirmation of pregnancy, the female was housed individually. Maternal weight and food and water consumption were monitored daily throughout pregnancy.

On day 6 of pregnancy, rats were randomly divided into four groups (n=12 per group): normoxic and hypoxic pregnancy, with or without allopurinol treatment (30mg.kg-1.d-1 in jelly). The dose of allopurinol used was derived from a previous study in our laboratory that achieved elevations in circulating allopurinol concentrations within the required range to be able to act as an antioxidant [37]. Furthermore, it was comparable to a dose of allopurinol given postnatally to severely asphyxiated neonates in order to improve postnatal outcome [38]. Hypoxia was induced by placing pregnant rats inside a chamber, which combined a PVC isolator (PFI Plastics Ltd., Keynes, UK) with a nitrogen generator. Pregnant rats subjected to hypoxia were maintained at a constant inspired fraction of oxygen of 13% from day 6 to 20 of gestation. The percentage of oxygen inside the chamber was controlled by altering the inflow of air and nitrogen. Oxygen concentration was monitored continuously throughout the treatment period with an oxygen analyzer (ICA, London, UK). The hypoxic chamber contained a separate sealed unit, which allowed food and water intake, and maternal weight, to be monitored daily throughout gestation.
At birth, each litter was sexed by measurement of ano-genital distance and pups were weighed and culled to eight, to standardize postnatal nutrition and maternal care within each litter.

Body weight was recorded daily from P0 to P7, every other day till P21, and then weekly until the study was complete. All pups remained with their mothers until weaning at postnatal day 21, after which they were housed in pairs under standard conditions. To control for within litter variation and sex differences, only one male pup per litter was used for this study.

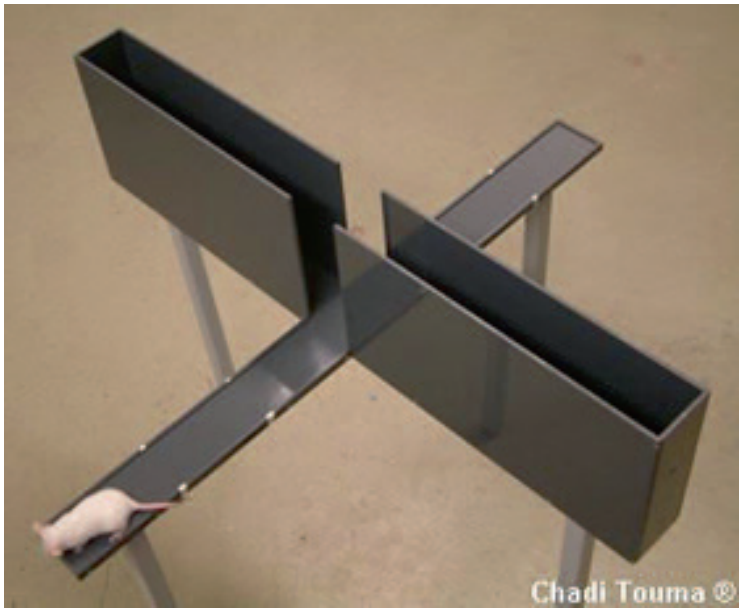
Behavioural tests

At 3.5 months of age, behaviour and cognitive function were assessed using an elevated plus maze (EPM), and object recognition (OR) task, respectively. All trials were run with the experimenter blind to the treatment conditions.

Elevated plus maze

This task relies upon rodents' proclivity toward enclosed spaces and fear of heights/open spaces and is considered a measure of locomotor activity, novel exploration, anxiety and risk taking behaviour [39-41]. The maze was made of black plexiglass and comprised two open arms (50 x 10 cm), and two closed arms of the same dimensions but with 0.5 cm high walls. The maze was elevated 70 cm above the ground (Fig. 1). Rats were placed in the centre of the maze facing one of the closed arms and the movements were recorded for 5 minutes using a camera mounted above the maze. The following parameters were analyzed by two independent observers: (1) entries into the open arms; (2) entries into the closed arms; (3) time spent in the open arms; (4) time spent in the closed arms; (5) ratio between time spent in open arms versus time spent in closed arms. An entry was recorded when the rat entered an arm with all four paws. After testing each animal, the maze was cleaned with water to eliminate olfactory stimuli.

Figure 1. Elevated plus maze apparatus.



The maze was made of black plexiglass and comprised two open arms (50 x 10 cm) and two closed arms of the same dimensions but with 0.5 cm high walls. The maze was elevated 70 cm above the ground. Rats were placed in the centre of the maze facing one of the closed arms and their movements were recorded for 5 minutes using a camera, which was mounted over the maze. Image from the website of the Max Planck Instute of Psychiatry (http://www.mpipsykl.mpg.de).

Object recognition

The object recognition memory task is mediated by non-hippocampal structures, mainly the perirhinal cortex [42-45]. It is a test to assess a form of declarative memory. The OR task is based on rat's spontaneous tendency to explore novelty in their environment [44,46-48]. The OR task was conducted in an Y-shaped apparatus, with walls to prevent the rat from looking out, thereby,

maximizing his attention to the object stimuli. The apparatus (Fig. 2) was raised 30 cm above the floor with walls 40 cm high. Each arm was 27 cm in length and [44] 10 cm wide. A video camera was mounted above the apparatus to record all trials. To prevent the objects from being displaced during a trial, they were stuck to the floor of the apparatus with Blu Tack (Bostik, Stafford, UK). The floor and walls of the apparatus were wiped down with a dry paper towel between trials.

Figure 2. Object Recognition apparatus.

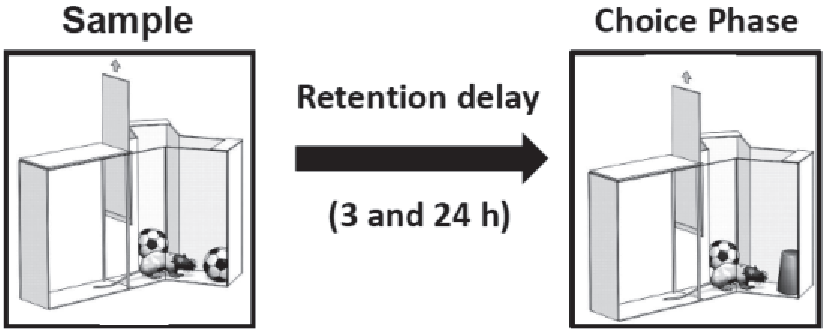


Image showing the Y-maze apparatus utilised for the Object Recognition task. The apparatus is raised 30 cm above the floor with walls 40 cm high. Each arm was 27 cm in length and 10 cm wide. The start arm contains a guillotine door. A video camera was mounted. In the sample phase, the rat is exposed to identical versions of the same object, one at the end of each exploration arm. The rat explores these objects for 3 minutes being removed from the apparatus for the 3 or 24 hour retention delay. Following the retention delay, the rat is reintroduced to the apparatus, which now contains an identical copy of the sample object at the end of one exploration arm and a novel object at the end of the other arm. Image from Winters et al. [44].

All rats were habituated for 5 min in the empty Y-apparatus on two consecutive days prior to testing. Each trial consisted of two phases. In the sample phase, two identical objects were placed in the Y-shaped apparatus, one at the end of each exploration arm. The rat was placed in the start box. The guillotine door was then raised to allow the rat to enter the maze. When the rat exited the start box the sample phase began. Exploration of an object was defined as reaching to it with the nose within 2 cm of the object. Turning around or sitting on the object were not considered exploratory behaviour. After 3 min the rat was removed from the apparatus for the duration of the retention delay. After the delay, the rat was placed back in the start box and released into the exploration area for the choice phase. The apparatus now contained the sample (familiar) object in one arm and a new object in the other arm. The arms in which the choice objects were placed were counterbalanced between rats and across trials. The rat was allowed to explore the objects for 3 min, at the end of which it was removed and returned to its home cage. The time spent exploring each object was assessed from video recordings. Data were collected using ODLog (Macropod Software, US). The time spent exploring the novel and familiar objects was assessed for 3 min, but attention was focused on the first minute, during which object discrimination is typically greatest [44,49]. A discrimination ratio (DR) was used as a measure of memory consolidation where DR = (n-f)/(n+f), with 'n' being the time spent exploring the novel object, and 'f' being the time spent exploring the familiar object. We calculated a DR for the first

minute of the choice phase on each object recognition trial. This measure takes into account individual differences in the total amount of exploration time. Furthermore we calculated total time exploring in the first minute for both the 3 and 24 hours retention delay. Animals that were exploring < 10 sec in 3 minutes were excluded from analysis.
At 4 months of age, rats were deeply anaesthetised (1ml Euthanal®, sodium pentobarbital), then perfusion fixed (4% PFA) for biometry and tissue collection. Tissues will be used for future histological analysis to assess cerebral development and injury

Statistical Analysis

Data are presented as mean ± SEM. Data were analysed by one-Way ANOVA followed by the Tukey post hoc test. SigmaStat software (SigmaStat for Windows, Version 2.0) was used for all statistical analyses. P values <0.05 were accepted as statistically significant.

Results

Biometry

Body weight at birth and at 4 months of age was not different between normoxic and hypoxic pregnancies (P>0.05, Table 1). At 4 months of age, absolute and relative brain, liver and heart weights, and ponderal index (PI), were not significantly altered between normoxic and hypoxic pregnancies (P>0.05). Relative to normoxic offspring, the brain to liver weight ratio was significantly increased and crown rump length (CRL) decreased, in offspring of hypoxic pregnancies (both P<0.05). Maternal treatment with allopurinol in hypoxic pregnancies did not prevent the decrease in CRL. Maternal treatment with allopurinol in normoxic and hypoxic pregnancies did not result in other significant alterations in biometric indices (P>0.05).

Table 1. Biometry data

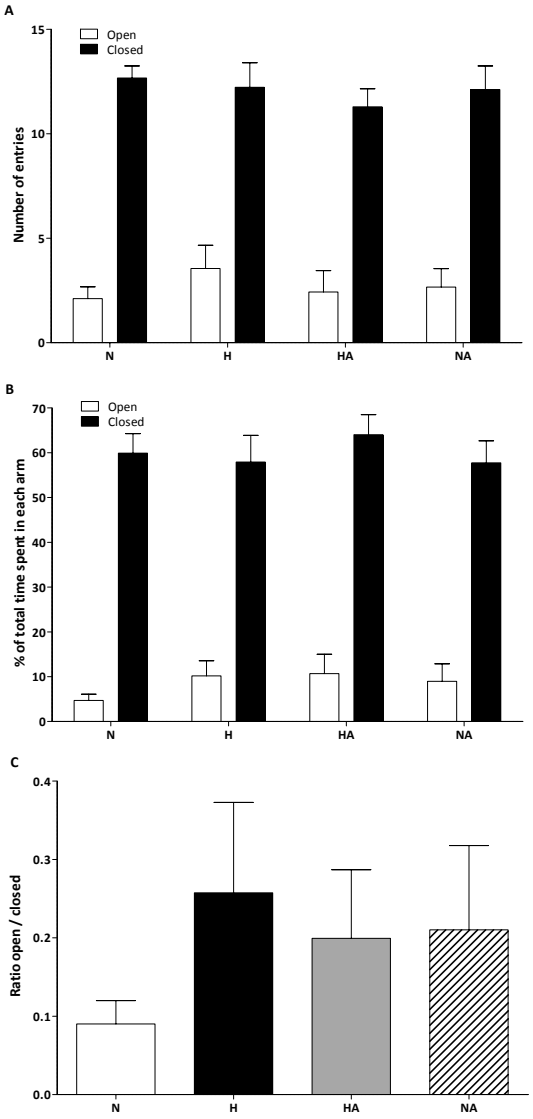
	N (N=11)	H (N=10)	HA (N=9)	NA (N=10)
	Postnatal day 0			
Birth weight (g)	6.40±0.14	6.56±0.10	6.20±0.17	6.21±0.17
	16 weeks after birth			
Body weight (g)	567±12	530±14	542±21	560±11
CRL (cm)	23.5±0.24	22.3±0.10*#	22.7±0.21*	23.4±0.22
Brain weight (g)	2.05±0.03	2.05±0.04	2.02±0.03	2.08±0.04
Brain/body weight (%)	0.36±0.007	0.39±0.008	0.38±0.016	0.37±0.012
Heart weight	1.75±0.05	1.57±0.05	1.64±0.06	1.71±0.05
Heart/body weight (%)	0.31±0.007	0.30±0.007	0.30±0.010	0.31±0.009
Liver weight (g)	23.0±0.68	18.9±0.68	20.8±1.05	22.0±0.72
Liver/body weight (%)	4.06±0.07	3.56±0.07	3.83±0.08	3.94±0.14
PI (BW/CRL^3)	0.044±0.001	0.048±0.001	0.047±0.002	0.044±0.002
Brain/liver weight (g/g^{-1})	0.090±0.003	0.109±0.003*	0.099±0.005	0.095±0.004

Weight at birth and biometry data at 16 weeks of postnatal age of the four groups of animals used for behavioural testing. *P<0.05 vs normoxic, #P<0.05 vs normoxic+allopurinol (One-Way ANOVA+Tukey test).

Elevated plus maze

Anxiety-related, explorative behaviour and locomotor function, as assessed by the elevated plus maze, was not significantly altered in hypoxic offspring relative to normoxic offspring (P>0.05; Fig. 3). Maternal treatment with allopurinol in normoxic and hypoxic pregnancies also did not result in significant alterations in performance (P>0.05; Fig. 3).

Figure 3. Elevated plus maze.

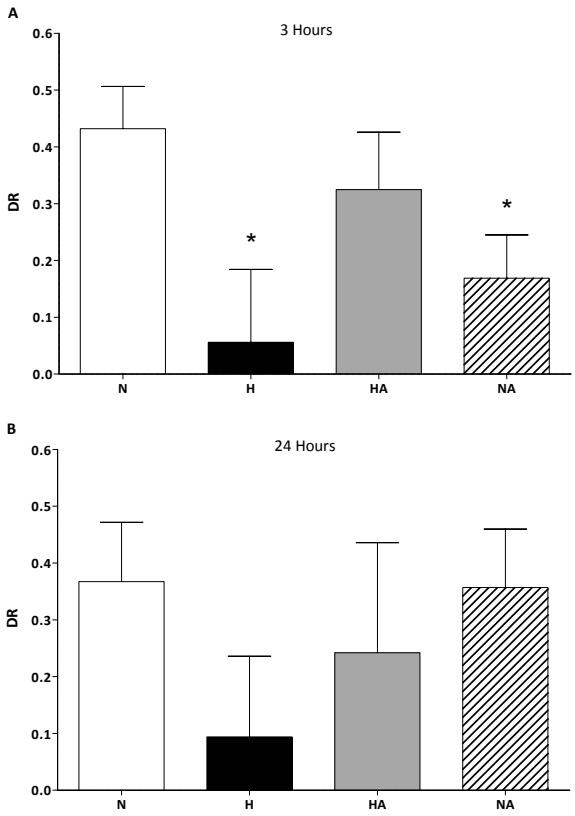


(A) The number of entries (B) the time spent in each arm (C) the ratio spent in open versus closed arms by normoxic (N, n=9), hypoxic (H, n=9), hypoxic + allopurinol (HA, n=7), and normoxic + allopurinol (NA, n=9) offspring. P>0.05 (One-way ANOVA).

Object recognition

There was no significant difference between the groups in the time required to complete 25 seconds of exploration in the sample phase, which suggests no adverse effects of hypoxia on exploratory behaviour (3 hour sample phase; N: 78.8±14.2, H: 65.2±7.9, HA: 63.7±5.4, NA: 52.7±6.2 sec; P>0.05; 24 hour sample phase; N: 128.2±12.4, H: 104.3±14.8, HA: 108.5±26.5, NA: 135.2±14.7 sec; P>0.05). Relative to offspring of normoxic pregnancies, the discrimination ratio (DR) was significantly reduced in offspring of hypoxic pregnancy 3 hours after testing (Fig. 4A; P<0.05). The DR in the first minute remained attenuated 24 hours after testing in offspring of hypoxic pregnancy however it did not approach significance (Fig. 4B; P>0.05). These adverse effects on cognition were absent in offspring of hypoxic pregnancy treated with allopurinol (Fig. 4 A,B;P>0.05). Interestingly, treatment of normoxic pregnancy with allopurinol significantly reduced the DR 3 hours after testing (Fig. 4 A; P<0.05).

Figure 4. Object Recognition task.



The discrimination ratio (DR) at (A) 3 hours and (B) 24 hours following the sample phase in normoxic (N, n=9), hypoxic (H, A: n=8; B: n=10), hypoxic + allopurinol (HA, A: n=7; B: n=6), and normoxic + allopurinol (NA, n=9) offspring.* P<0.05 vs. normoxic (One-way ANOVA + post hoc Tukey test).

Discussion

The data show that chronic fetal hypoxia resulted in impaired object recognition in adulthood, an effect that can be ameliorated by maternal treatment with allopurinol. Maternal allopurinol treatment in healthy pregnancy appeared to delay the initial stages of memory encoding. Chronic fetal hypoxia did not induce anxiety-related or locomotor behaviour.

We showed an impairment in object recognition memory in 3.5 months old adult offspring from hypoxic pregnancies. This replicates previous studies that have shown cognitive impairment by inducing mild hypoxia using a similar model. Janicke et al., induced mild chronic hypoxia (12%O2) during gestational days 0-17 in rats and observed in juvenile rats a delay in learning ability of hypoxic offspring, in that they oriented themselves less successfully in the olfactory test and water maze [50]. These deficits disappeared during the adult phase [50]. Maternal food intake was equal in hypoxic and normoxic pregnancy in this study [50], however water intake was decreased in hypoxic pregnancies. A study in mice compared offspring of hypoxic pregnancy, induced for 2 hours in a hypoxic chamber (9%) at embryonic day 17, to offspring of normoxic pregnancy. Spatial learning in hypoxic offspring, as examined in the Morris water maze, was slightly slower and memory for the platform's location was poorer, when tested at 4 and 6 months of age [51]. However, maternal food and water intake were not reported. When pregnant rats were exposed for 3 hours to 7% oxygen from embryonic day 14 or 18, impairments in long-term and short-term memory were observed in adult offspring [52]. Maternal food intake was not reported in this study. Hypoxia in late embryonic development in a chick model (10% hypoxia from embryonic day 14 to 18) reduced long term memory of an aversive stimulus the first days postnatal using the bead task [14]. Additionally, in other studies which have induced placental insufficiency through uterine artery or umbilical cord clamping have shown similar impairments in cognitive function [45,53-57], however, these insults reduce both oxygen and nutrient supply, thus making it difficult to determine the isolated effects of hypoxia.

The object recognition task comprises three stages; sample presentation (encoding/acquisition), the choice phase (retrieval), and the retention interval (storage/consolidation) [46]. The perirhinal cortex is known to be crucial for object recognition [58-65]. Many studies have implicated cholinergic input to the perirhinal cortex as playing a major role in object recognition memory [65-69]. Winters et al. observed a direct role for cortical acetylcholine in the acquisition, but not consolidation or retrieval, of OR [69]. The N-methyl-D-aspartate (NMDA) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors within the perirhinal cortex have been shown to be involved in both acquisition and consolidation of the object memory [64]. NMDA and AMPA receptors contribute differentially to synaptic transmission and both are important for aspects of synaptic plasticity [70-72]. Following a prenatal insult such as heroin exposure in mice [73], glucocorticoid treatment in guinea pigs [74] and hypoxia in sheep [75], changes in the cholinergic system and the number of NMDA receptors have been observed. Interestingly, a previous study in our laboratory has shown that chronic hypoxia for the same duration as

utilised in the current study significantly reduces neuronal number, vascularity and synaptophysin in the hippocampus and reduces vascularity in the perirhinal cortex (Camm et al., manuscript in preparation). We have seen in our model more cyto-architectural changes in the hippocampus rather than in the perirhinal cortex. Therefore, it is likely that damage as a result of chronic fetal hypoxia may be neurotransmitter-related, or connectivity between the perirhinal cortex and the hippocampus is altered in our model.

Results in the present study not only confirm the deleterious effects of chronic fetal hypoxia on adult behaviour, but they also extend these findings to show that maternal treatment with allopurinol in hypoxic pregnancy prevented these programming effects. It is being shown that prenatal hypoxia can increase ROS and generate oxidative stress in the placenta [21], cardiovascular system [19,76-80], liver [81,82] and the lung [81]. Oxidant molecules can directly interact with DNA base pairs causing both genetic, as well as, epigenetic changes, the latter through alterations in DNA methylation and histone modification [83]. Intrauterine hypoxia and IUGR have been shown to alter DNA methylation of selected genes in several tissues including placenta [84], heart [85,86], liver [87] and adrenal gland [88]. Therefore, oxidative stress has been ascertained to be a contributing factor in epigenetic mechanisms [30,89]. Allopurinol is acting as a xanthine oxidase inhibitor and free radical scavenger [31,32,36] can reduce the formation of free radicals during hypoxia [31,33] and thereby limit damage and preventing changes in fetal programming causing memory impairment. The implications of these data are that maternal treatment with antioxidants may provide possible therapy against the programming effects on neurological dysfunction in pregnancy complicated by fetal hypoxia, such as during placental insufficiency or high altitude pregnancy.

Interestingly, maternal treatment with allopurinol in normoxic pregnancy also led to offspring with decreased object recognition memory. Maternal antioxidant supplementation may therefore only restore the offspring in pregnancy conditions associated with increased superoxide generation and oxidative stress. Antioxidant treatment in healthy conditions where the offspring is already replenished with an appropriate redox balance may, in fact, lead to oxidative stress. This replicates previous studies that have observed adverse effects of antioxidants in healthy individuals. In healthy pigs, doses of vitamins C and E, which had been shown to reduce oxidative stress under pathological conditions, were found to increase oxidative stress in the kidney and heart [90,91].

Another aim of this study was to detect if prenatal hypoxia would induce anxiety and/or risk taking behaviour, and if so, if allopurinol could reduce this behaviour. Hypoxia during gestation sensitizes the hypothalamic-pituitary-adrenal-axis and might therefore be able to induce anxiety-like behaviour in adult offspring [92]. At 3.5 months of age we did not see any changes in anxiety related behaviour or locomotor function. Previous studies have shown that gestational hypoxia can transiently affect activity in juvenile animals [93] but this effects disappears in adulthood.

In a previous behavioural study using the same rat model, we have observed impairments in spatial memory using the Morris Water Maze, which implicates changes in the hippocampus (Camm et al., manuscript in preparation). This current study suggests that not only the hippocampus is impaired in this model, but also the perirhinal cortex.

Previous studies in our laboratory using the same model as used for this observed that early onset chronic hypoxia increased placental weight [19,21]. In our experimental rat model, placentation occurred during hypoxia as in rats, uterine wall invasion and the establishment of the placenta commences after day 9.25 of gestation. Consequently, early onset hypoxia may stimulate greater than normal placental growth, and therefore cushion the adverse effects on fetal growth. Accordingly, studies of human pregnancy at high altitude have reported greater than normal placental growth accompanied by improved placental vascularisation, increased placental capillary diameter, capillary length and capillary volume [94,95].

Comparing biometric data at 4 months postnatal age revealed no differences in body and brain weight, however, in hypoxic offspring, CRL was reduced, and the brain/liver weight ratio increased, which is likely due to the reduced liver weights in these offspring. The functional consequence of the reduced liver weight is currently being investigated.

Conclusions

This preliminary study has shown that object recognition memory is impaired in offspring of hypoxic pregnancy. Combined with our previous study, the data suggest that hypoxia can program cognitive impairment in later life. Further analyses are required to assess the structural correlate of this impairment. Treatment with allopurinol in hypoxic pregnancies improved cognitive function, further supporting the role of oxidative stress in developing programming. However, antioxidant treatment in healthy pregnancy may be detrimental and is not to be recommended.

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Chapter 9

Summary and general discussion

The clinical use of glucocorticoids before or after birth in case of threatened preterm birth or in preterm neonates has become common practice following the pioneering work of Liggins who discovered that a prepartum rise in fetal endogenous glucocorticoids is essential for the transition from intra- to extra-uterine life and that exposure to synthetic glucocorticoids in premature individuals could support pulmonary maturation (Chapter 1; [1,2]. It is now well established that pre- [3] and postnatal [4,5] glucocorticoids are a life-saving therapy for prematurely born infants. However, glucocorticoids also trigger unwanted side-effects. Accumulating evidence suggests that one pathway by which glucocorticoids may promote their deleterious effects is increasing oxidative stress and decreasing nitric oxide (NO) bioavailability. Increased rate of production combined with decreased elimination of reactive oxygen species (ROS) by dexamethasone leads to oxidative stress whereby ROS can damage cellular components, particularly in the delicate newborn period [6]. Given the fact that ROS react with NO to increase vascular tone [7,8] it is possible that the adverse effects of glucocorticoids are secondary to depleted bioavailability of NO [7,9].

The aims of this thesis were (*chapter 1*):

1. To elucidate the adverse effects of ante- and postnatal glucocorticoid therapy *(chapter 2-7)*.
2. To investigate whether a human clinically-relevant dosing regimen of postnatal glucocorticoid therapy increases oxidative stress and decreases NO bioavailability and whether combined treatment with the antioxidant vitamins C and E or the NO bioavailability increasing-drug pravastatin, ameliorates the detrimental effects of dexamethasone on somatic growth, cardiovascular function and brain development, all in rats *(chapter 4-6)*.
3. To explore another field where antioxidant treatment might be beneficial, namely in chronic fetal hypoxia (*chapter 8*).

The information gained from these studies may be important for obstetricians and neonatologists who are counseling patients about treatment in the perinatal period and who should weigh advantages and disadvantages of glucocorticoid treatment. In the various chapters of this thesis the results of the studies have been discussed extensively. Therefore the main aim of this final chapter was to give a brief overview of the most important results and discuss the major implications of these findings for the clinical situation. Future directions and recommendations that remain from the findings in the previous chapters will be also stated in this chapter.

PART I

Effects of antenatal glucocorticoid treatment on the hippocampus throughout development in mice and shortly after birth in human preterm neonates

The hippocampus is known to be particular sensitive to glucocorticoids because of the high expression of glucocorticoid receptors and mineralocorticoid receptors. In *chapter 2* we focused on the effects of a clinically relevant single antenatal dexamethasone treatment on the

development of the mouse hippocampus. We studied the effects of dexamethasone treatment on body weight, hippocampal volume, number of neurons and apoptotic cells in the CA and DG and proliferative cells in the subgranular zone (SGZ) of the DG, at E16, E18, P0, P5, P10, P20 and adult stage (6 months). Shortly after dexamethasone treatment the number of apoptotic cells was increased in both the CA and DG and proliferation was reduced in the SGZ of the DG of the hippocampus. This was followed by enhanced proliferation postnatally, but at adulthood, the number of proliferative cells was lower than in the control group. Body weight, hippocampal volume and the total number of neurons in the CA and DG were reduced by dexamethasone administration, but these effects were transient and did not persist in adulthood.

These findings demonstrate that a single antenatal dexamethasone treatment administered during a period of life when the brain is developing, has considerable adverse long-term effects in mice. These results are also suggestive for long-term adverse effects of antenatal glucocorticoids on the human brain. However, we should be careful when extrapolating data from animal models to the human situation, since major differences may exist between species [10]. The general sequence of brain growth is similar among species, however an important consideration is that the maximum velocity of brain growth in mice occurs after birth, in contrast to humans, in whom the maximum velocity of brain growth occurs around the time of parturition. The age at which the pups were exposed to dexamethasone treatment was at E15.5 (term E20), which is quite early in terms of brain development and perhaps more comparable with human hippocampal development in the second trimester of gestation (http://www.translatingtime.net/tables). Another important aspect is that animal models provide simplified observations without the vagaries of preterm birth and maternal disease, which do occur in the human situation. Moreover, sensitivity for glucocorticoids during development may be different, since rodents are thought to be more sensitive to glucocorticoids [11]. Therefore the results in mice might be more pronounced than in the human and it remains to be established whether adverse long-term effects of antenatal glucocorticoid treatment will be present in the human. In a previously published study using the same treatment protocol, our group showed impaired learning and hippocampal synaptic plasticity at the age of 6 months in mice [12]. Follow-up studies after antenatal administration of one course of glucocorticoids in human are thus far reassuring, with in only one study subtle neurological impairment showing at the age of 6 [13], but normal physical and physiological development at 12, 20 and 30 years [14-17]. However, if the adverse long-term effects of antenatal glucocorticoid treatment that we found in mice can be extrapolated to the human situation, than the effects may only be expressed at later stages in life. Moreover, delayed effects of antenatal glucocorticoid treatment in the human may only be revealed if the suitable parameters will be tested and analyzed. The follow-up studies mentioned before often measured intelligence to identify adverse effects of antenatal glucocorticoid treatment on brain function. However, the results described in *chapter 2* and data from other studies suggest that analysis of hippocampus-dependent memory and learning might reveal alterations in brain function after glucocorticoid treatment. Further detailed human follow-up is therefore recommended and should focus on hippocampal function, for instance spatial learning [10].

Because of the profound findings in *chapter 2* we investigated in *chapter 3* the relation between antenatal glucocorticoid treatment and hippocampal histology in human neonates. Included in this study were neonates with a gestational age between 24 and 32 weeks, who had died during or within 4 days after delivery and underwent brain autopsy. We observed a significantly lower density of neurons in the CA of the hippocampus of neonates who were treated with antenatal glucocorticoids. No difference was found in density of small neurons, in myelination, gliosis, proliferation, apoptosis or ischemic damage. The observation of a decreased neuronal density in the CA was consistent with the findings in *chapter 2,* showing a decreased number of neurons in the CA region of the hippocampus directly after treatment until postnatal day 10 and with earlier investigations using animal models [18-19]. An important limitation of this study was the heterogeneity of our patient group. Several pathological conditions during pregnancy, delivery and early neonatal life may have caused hypoxic ischemic insults before or after birth which may already had damaged the hippocampus. However, these potentially confounding effects can only be ruled out in a large cohort study with a controlled prospective design. For obvious reasons it will not be possible to realize such a study in human neonates. We tried to minimize confounding factors in several ways. First, by including only neonates, who lived for a maximum of four days, limiting influences of early neonatal life. Furthermore, we compared groups for indicators of recent damage, by performing a staining with acid fuchsin-thionin, which highlights neurons with recent hypoxic ischemic damage. No significant differences were found between the two groups. The functional consequences or long-term effects of the decreased neuronal density are hard to predict because effects may be transient and the plasticity of the brain at this age may perhaps compensate for a decrease in neurons in the hippocampus. However it might be that this is the first sign confirming our findings in *chapter 2*, indicating decreased neuronal reserves or it could be an indication of lasting less neurons in the CA. Above all it draws attention that more detailed and specific follow-up studies in humans are required.

Both, dexamethasone and betamethasone are used in clinical practice in case of imminent preterm delivery. They have similar biological activity and cross the placenta. Betamethasone has been suggested to be preferred over dexamethasone, since stronger beneficial effects have been observed after betamethasone treatment [20,21]. Unluckily however, betamethasone also has more direct side effects on fetal behaviour and fetal heart rate variation in the human fetus [22]. Therefore, we probably may expect that long-term side effects will also be more pronounced after betamethasone than after dexamethasone. Furthermore, the dose of glucocorticoids could be questioned as, since the introduction of glucocorticoid treatment in preterm infants 40 years ago, there has been no modification of the human clinical dosing regimens. This is alarming, considering the fact that the clinical dosing regimen has been adopted from the experimental studies of Liggins (1969) [1], which were designed to promote preterm labour in sheep and, incidentally, demonstrated that steroids also stimulate lung maturation.

A treatment that specifically targets the lung without having side effects on other organs would be an ideal alternative. However until new treatments to enhance fetal lung development are available obstetricians should be cautious with the use of glucocorticoids, and multiple courses should be avoided since adverse effects are dose related. The use of glucocorticoids may be

kept to a minimum by applying the correct identification of preterm labour, which is nowadays a hot topic in perinatal research and facilitated by cervical length and fibronectin measurements [23,24].

PART II

In part II the focus was on postnatal glucocorticoid treatment. As mentioned before, we hypothesized that generation of ROS and depleted NO bioavailability are part of the mechanism causing adverse effects observed with postnatal glucocorticoid treatment. In *chapter 4* we reported on the effects of a clinically relevant dose of dexamethasone on three molecular indices of oxidative stress in the rat brain at postnatal day (P) 21. Dexamethasone treatment led to a significant increase in lipid peroxidation (increased 4-hydroxynonenal content), in expression of heat-shock protein 70 (Hsp70) and of nitrotyrosine (NT) in the cortex. Addition of the antioxidant vitamins C and E to dexamethasone treatment was used in order to decrease oxidative stress. Vitamin C, also known as ascorbic acid, is believed to be the most effective aqueous-phase antioxidant in human plasma because of its ability to trap peroxyl radicals, thereby preventing lipid peroxidation [25]. Vitamin E, also known as α-tocopherol, is one of the most important inhibitors of the free-radical chain reaction of lipid peroxidation because of its peroxyl radical scavenger capacity, giving it a major role in protecting biological membranes [26]. Co-treatment with vitamins C and E restored Hsp70 protein expression induced by dexamethasone treatment; however, the up-regulation of 4-HNE and NT remained unchanged. The data in *chapter 4* therefore confirm that clinically-relevant doses of dexamethasone induce oxidative stress in the developing brain and that antioxidant therapy with vitamins C and E only partially protect against this effect.

In *chapter 5 and 6* we used the same dosing regimen for dexamethasone as in *chapter 4* but this time in combination with pravastatin. Stastins, or the group of HMG-CoA reductase inhibitors, have been shown to have 'pleiotropic' effects in addition to their lipid lowering actions, including reductions in platelet aggregation [27], decreases in arterial stiffness [28], and improvements in vascular endothelial tone [29]. All benefits that have been credited to increases in NO bioavailability [30-32]. There are several possible mechanisms through which pravastatin may lead to sustained increases in NO bioavailability, as discussed in *chapter 6*. Observations in *chapter 5 and 6,* corresponding with other published studies [9,33], show that administration of dexamethasone to postnatal rat pups led to significant decreases in plasma levels of nitrates and nitrites (NOx) at P21, well established indices of circulating NO bioavailability. Additional treatment with pravastatin normalized NOx levels. Data in *chapter 5 and 6* therefore confirm that clinically-relevant doses of dexamethasone reduce NO bioavalability and that pravastatin is effective in protecting against this effect.

Effects of postnatal glucocorticoid treatment on growth

In *chapter 4, 5 and 6* we also investigated effects of a clinically relevant human postnatal dosing regimen of dexamethasone on body weight, organ weights, crown rump length (CRL), ponderal index and foot length in rats. We found that dexamethasone treatment of rat pups decreased body weight gain, an effect that was already evident 24 h following the beginning of treatment and continued until P21 (*chapter 6*). Fractional growth rate (FGR) is an useful measure to assess relative growth between groups, it relates weight gain to initial body weight over a specific period of time. In control pups, FGR was highest during P0-7 and subsequently decreased during P9-14. Conversely, in dexamethasone treated pups, FGR was markedly reduced from P0-7 and increased during P9-P14. The increase in FGR in the week following treatment may have indicated accelerated or 'catch-up' growth that occurred once the suppressive effect of dexamethasone was removed. Postnatal 'catch-up' growth can occur in intrauterine growth restricted babies and is an important risk factor for subsequent hypertension, heart disease, and insulin resistance [34]. The 'catch-up' growth that we observed after postnatal dexamethasone treatment may therefore have important consequences on the development and the expression of disease later in life. As this was an important finding we studied in *chapter 7* the effects of postnatal glucocorticoid treatment on somatic growth in human preterm infants. We compared longitudinal growth data (length, body weight and head circumference) of a reference preterm group with those of preterms treated with hydrocortisone or dexamethasone, using reference curves exclusively developed for preterm infants. This study showed a striking similarity with the results of the rat studies. Mainly growth velocity was affected and the most important finding was an effect on FGRs of weight, height and head circumference. FGRs were generally reduced in the dexamethasone and hydrocortisone treated groups during the first two months of age (the treatment period), with 'catch-up' growth in the following months. We therefore suggest that these treated children may have an increased health risk in later life and should be carefully monitored with respect to metabolic and cardiovascular diseases. Furthermore, the pattern of head circumference growth is highly related to neurodevelopmental outcome [35], with 'catch-up' growth in the first three months after birth being important for later neurodevelopmental outcome in very low birth weight infants (<1500g). This was exactly the period when FGRs were decreased in the infants treated with hydrocortisone or dexamethasone, and may suggest impaired neurodevelopmental outcome. Follow-up studies thus far have reported adverse effects of postnatal dexamethasone treatment on neuromotor and cognitive outcomes and [36-38]. Changes in growth patterns were more pronounced in dexamethasone than in hydrocortisone treated infants. This could be a consequence of a difference in risk between dexamethasone and hydrocortisone but it may also indicate overtreatment in the dexamethasone treated group, as the dose of dexamethasone used in *chapter 7* was 2.5 times more potent than the dose used for hydrocortisone [39]. The dexamethasone dose was, however, identical to that commonly used in the nineties.

The dexamethasone induced growth restriction in *chapter 5&6* was asymmetric, reflected by an increased brain to body weight ratio and by an increase in the ponderal index, the ratio of brain to liver weight and of CRL to foot length. This is interesting because, next to 'catch-up' growth,

asymmetric growth restriction [40] is an independent strong risk factor for the development of cardiovascular and metabolic diseases later in life [41]. In *chapter 7* we did not obtain information about the symmetry of growth, but this aspect will be further assessed in further studies. Both changes, 'catch-up' growth and asymmetric growth restriction, that we found in *chapter 4-7* warrant further follow up of preterm infants treated with postnatal glucocorticoids focusing on cardiovascular and metabolic diseases in later life.

Effects of dexamethasone treatment combined with vitamins C and E or pravastatin on growth

Dexamethasone treatment combined with vitamins C and E (*chapter 4*) also reduced body weight at weaning and had no beneficial effect on brain weight, which was comparable to dexamethasone treatment alone. In *chapter 5 and 6* pups were treated with dexamethasone and pravastatin. The dexamethasone-induced suppression of growth rate was ameliorated but body weight was not restored to control levels at P21 (comparable with the findings in *chapter 4)*. This suggests that increased NO bioavailability may improve growth, for instance via increasing perfusion and the delivery of oxygen and nutrients [42] and/or promoting angiogenesis [43,44]. However, these effects are not strong enough to revert the powerful stunting effects of steroids in the postnatal period. FGR in the dexamethasone and pravastatin treated pups was markedly reduced from P0-7 and increased from P9-14. Compared to dexamethasone treated animals, additional administration of pravastatin led to a further increase in FGR between P9-P14, indicating that also in the dexamethasone and statin treated animals there is also 'catch-up' growth.

The effects of postnatal glucocorticoid treatment on brain development with and without co-treatment with vitamins C and E or pravastatin

In *chapter 4 and 5* we also investigated the effects of a clinically relevant dosing regimen of postnatal dexamethasone treatment on brain development. We histologically and stereologically analysed the brains at P21, and found that relative to controls, dexamethasone decreased total brain volume, decreased soma volumes of neurons in the CA1 and in the dentate gyrus of the hippocampus (*chapter 4,5*). A reduction in neuronal soma volume may be related to a decrease in cellular function [45] or reflect shrinking of neurons, often preceding neurodegeneration [46]. The decrease could further indicate a preferential loss of larger neurons, which has been observed in neurodegenerative diseases [47], or it could be an indication of a delay in neuronal differentiation and/or dendritic growth [48]. In *chapter 5* we reported a decreased number of cortical neurons, however in *chapter 4* the number of cortical neurons was not significantly different. The apparent discrepancy between the number of cortical neurons observed in *chapter 4 and 5* might be caused by the number of animals that we analyzed (chapter 4: n=5 per group, chapter 5; n=7/8 per treatment group). Dexamethasone-dependent reductions in cortical neuronal number may be due to a suppression of neurogenesis as reported before, following corticosterone treatment in rats [49,50], or to impaired neuronal proliferation [51] or enhanced cell death [52], as both reported in rats following dexamethasone treatment. Furthermore we found an increased GFAP-

positive astrocyte density in the white matter after postnatal dexamethasone treatment. The consequences of our findings on the functioning of the brain were not were not explored in this study. Randomized clinical trials of postnatal dexamethasone therapy have reported an increase in the rates of cerebral palsy and adverse neuromotor and cognitive outcomes in dexamethasone treated infants [37,53].

The addition of antioxidant vitamins to dexamethasone treated pups (*chapter 4)* resulted in improvements in total brain volume and soma volume of neurons in the CA1 and the DG. The addition of pravastatin treatment was found to be protective against all adverse effects on brain development reported in *chapter 5* i.e. brain volume, average soma volume in CA 1 and DG, cortical neuronal number and astrocyte density in the white matter. This suggests that combined glucocorticoid and vitamins C and E/statin therapy may be safer in the preterm infant than glucocorticoid treatment alone.

The effects of postnatal glucocorticoid with and without pravastatin treatment on cardiovascular function

In *chapter 6*, significant elevations in basal arterial blood pressure at P21 were described after postnatal dexamethasone treatment. We found no alterations in basal heart rate at P21, and others using a similar model found no alterations of cardiac output at 4 weeks of age [54]. This suggests an effect of postnatal dexamethasone on peripheral vascular resistance as the main mechanism contributing to hypertension.

Previous publications have already demonstrated several mechanisms by which glucocorticoids may induce an increase in peripheral vascular resistance: through increased sympathetic drive [55-57], enhanced responsiveness of the peripheral circulation to α_1-adrenergic agonists [56,58], endothelin [58-60] and angiotensin II [61,62] or impaired endothelial NO synthesis [33,63]. However, the peripheral vascular resistance may also be influenced by the vascular oxidant milieu [8,64], as vascular cells generate ROS, such as the superoxide anion [65]. Increases in superoxide will lead to a reduction in NO bioavailability, as superoxide will readily react with NO [8].

In *chapter 6,* postnatal administration of dexamethasone led to decreased levels of NOx (as reported in *chapter 5*), to enhanced femoral constrictor reactivity to phenylephrine and to depressed femoral dilator responsiveness to endothelium-independent and dependent agonists, both at P21. Concomitant treatment with pravastatin in dexamethasone-treated pups restored the amount of circulating plasma NOx to control values at p21, as did the peripheral vasculature constrictor and dilator reactivity. Therefore, the data presented in *chapter 6* support the concept that glucocorticoids affect vascular function and arterial blood pressure by decreasing NO bioavailability in the peripheral vasculature, and that pravastatin restores NO bioavailability, resulting in a normalization of the vascular oxidant tone.

We also found that the rate-pressure product was raised in dexamethasone-treated pups, indicating a higher myocardial workload in comparison to control animals [66]. An increased afterload is likely to have cause this effect, resulting in hypertension and shifts in the peripheral vasculature towards a constrictor phenotype. Concomitant postnatal pravastatin in dexamethasone-treated pups may therefore restore the rate-pressure product at P21 by increasing NO bioavailability and

normalizing peripheral vascular reactivity; effects which have been described in *chapter 6*.
The rate-pressure product at P21 may therefore be restored by increasing NO bioavailability and normalising peripheral vascular reactivity in concomitant postnatal pravastatin in dexamethasone-treated pups. These effects were described in *chapter 6*.
Despite the hypertension observed in vivo and the increase in constrictor vascular reactivity in vitro, dexamethasone treated hearts displayed impaired pressure generating contractile ability in vitro, associated with impaired systolic and diastolic function and with reductions in coronary flow rate. An increase in peripheral vascular resistance may be a compensatory mechanism in the light of a failing heart. Alternatively, an increase in cardiac afterload may induce secondary cardiac failure. It has previously been demonstrated that left ventricular thickness is decreased in this model of dexamethasone administration [67] with impaired systolic contraction and increased end-diastolic volume in vivo was observed [54]. Acute and chronic alterations in autonomic control following glucocorticoid administration have been reported in both animal models and humans. Furthermore, clinical studies have shown that glucocorticoids administration decreases heart rate variability both in utero [68-71] and postnatally [72].
In *chapter 6* we have showed that neonatal dexamethasone treatment significantly decreased fetal heart rate variation and led to a significant increase in the low frequency/high frequency ratio in the frequency domain. Combined, past and present data support the underlying idea that glucocorticoids programme a shift in autonomic balance from parasympathetic towards sympathetic dominance.
All markers of heart rate variation were restored to control levels in dexamethasone-treated pups that were concomitantly treated with pravastatin. These results suggest that depletion of NO and alterations in redox state during development may contribute to the development of basal autonomic control of the circulation, what makes pravastatin co-treatment a promising therapy in decreasing the adverse effects of postnatal dexamethasone treatment.

Clinical implications of part II

This part of the thesis provides evidence for adverse effects of postnatal glucocorticoid therapy on neonatal development. As there is rising concern about the unwanted side effects of dexamethasone administration in the postnatal period, there is debate surrounding the choice and dosing strategies of glucocorticoids in premature infants. Because hydrocortisone is known to have less adverse effects than dexamethasone, hydrocortisone it is often chosen as the drug of choice. Conversely, as a result of meta-analyses of randomized controlled trials dexamethasone is known to offer greater beneficial effects on respiratory function than hydrocortisone [4,5,73]. However it should be noted that the meta-analysis of dexamethasone and placebo did not use similar outcome measures as the ones comparing hydrocortisone with placebo. Interestingly, retrospective studies have reported on favorable effects of HC in terms of weaning from the ventilator, need for extra oxygen and facilitation of extubation [74,75]. Unfortunately, evidence from randomized trials comparing effects of dexamethasone and hydrocortisone is lacking [73]. Greater harmful and beneficial effects of dexamethasone over hydrocortisone are understandable, since dexamethasone has a 25 times stronger relative potency and has a

longer half-life in comparison to hydrocortisone [76]. As long as its detrimental effects could be controlled without affecting its maturational effects, the greater beneficial effects on respiratory function of dexamethasone would therefore make it the glucocorticoid of choice in clinical practice. Potential ways for modification of current glucocorticoid therapy have concentrated on studies investigating the effects of lower doses [77] or altering the route of administration [78,79]. As stated before, modification of perinatal glucocorticoid treatment to remove unwanted side-effects, is only promising in a clinical setting if the alterations to standard practice do not prevent the beneficial effects of glucocorticoids on lung maturation. Treatment of preterm neonates with glucocorticoids in either the pre- or postnatal period is based on the fact that they reduce inflammation and promote surfactant mediated increases in functional residual capacity [80-82]. That they induce alveolar wall thinning, increasing air space volume whilst decreasing tissue volume and increase lung antioxidant capacity and accelerate lung fluid reabsorption [81,83,84]. In *chapter 6*, dexamethasone treatment with or without pravastatin led to significant increases in the expression of surfactant protein C in the lungs of pups at P21. While concomitant pravastatin treatment significantly diminished the adverse consequences of dexamethasone on the cardiovascular system and on brain development (*chapter 5*), it did not affect the established effect of the steroid on pulmonary surfactant synthesis [85,86]. Data in *chapter 5 and 6* showed that concomitant treatment of pups in the postnatal period with dexamethasone and pravastatin diminish the unwanted side-effects of the glucocorticoid on development of the cardiovascular system and brain while maintaining the induction of pulmonary surfactant. Therefore, our data suggest that the use of statin therapy in conjunction with glucocorticoids in premature infants may be safer than administration of glucocorticoids alone. We have not investigated the induction of pulmonary surfactant production in dexamethasone combined with antioxidant vitamins C and E yet; this will be subject of further study.

Some of the adverse consequences of postnatal dexamethasone treatment reported have already been verified in human clinical studies; however, caution is necessary when extrapolating data to the human when there is evidence from only animal studies, since the sensitivity for glucocorticoids among species may be different during development [11].

Treatment with vitamins C and E or pravastatin in healthy individuals

Antioxidant vitamins or pravastatin supplementation may improve disease states or conditions associated with enhanced free radical generation. However antioxidant treatment in healthy conditions where the physiology of the individual is already replenished with an appropriate concentration of antioxidants, may, in fact, lead to excess antioxidant capacity and excess NO bioavailability. The latter will promote peroxynitrite generation, thereby mimicking oxidative stress with subsequent induction of a number of unwanted side-effects [87,88]. Therefore, another objective of this thesis was to determine the isolated effects of antioxidant treatment with vitamins C and E and pravastatin *(chapter 4-6)* in control rats. We observed that treatment of rat pups with vitamins C and E alone increased Hsp70 and 4-HNE (well known indicators of oxidative stress) in the cortex, and NT in both the cortex and hippocampus. Interestingly, postnatal pravastatin treatment alone caused both endothelial-dependent and -independent vasodilator dysfunction

in femoral arteries at P21, and was associated with a decrease in plasma NOx levels. Whilst an increase in oxidant tone is known to be detrimental, chronic reductions in ROS generation may also have long-lasting negative effects as free radicals are involved in physiological signaling mechanisms [89]. By increasing NO bioavailability, pravastatin treatment may down-regulate elements of control of the vascular smooth muscle contractility. Such mechanisms could explain the reduced femoral vascular smooth muscle relaxation sensitivity and plasma NOx levels at P21 following pravastatin treatment alone in *chapter 6*. Therefore antioxidant supplementation is not recommended in healthy individuals.

Interestingly, treatment of pups with pravastatin alone also led to a significant increase in pulmonary surfactant protein C expression. Previously, in a mouse model of chronic airway inflammation, Ahmad and others also have reported that in sensitised lung tissue simvastatin was able to increase the expression of endothelial nitric oxide synthase (eNOS), to reduce NT staining and reduce asymmetric dimethyl-arginine, an endogenous inhibitor of eNOS [90,91]. Furthermore, inhaled NO therapy improves lung function and reduces oxygen dependence in neonates moderately, but not severely affected with bronchopulmonary dysplasia [92-95]. So, the mechanism mediating the improved respiratory function found with NO treatment is not only a reduction in pulmonary vascular resistance, as cultured human type II alveolar cells exposed to NO have an activated soluble guanylyl cyclase pathway and increased surfactant protein C mRNA expression [96]. Therefore, it is very well possible that additional pleiotropic effects of pravastatin on lung function are mediated through increases in NO bioavailability in pulmonary tissue.

PART III

Antioxidants in chronic fetal hypoxia

In *chapter 8* we investigated another indication for antioxidant therapy. In a preliminary study we reported the effects of chronic fetal hypoxia on behaviour and cognitive function in rat during adulthood, and determined whether the xanthine-oxidase inhibitor allopurinol had any neuroprotective effects. Compared to control rats, short term object recognition memory was significantly reduced in adult offspring of hypoxic pregnancy. Object recognition remained attenuated 24 hours after testing in offspring of hypoxic pregnancy, suggesting long-term memory impairment. Treatment with allopurinol in hypoxic pregnancies improved cognitive function. Anxiety-related behavior, risk taking behaviour and locomotor function were not different between the groups. Antioxidant treatment might be a promising therapy in the future in hypoxic pregnancy. This study is part of a much larger study that comprises also investigations on cerebral structure. The results of this preliminary study should be interpreted with caution and further analyses are required to work out the structural correlate of this.

Future directions and recommendations

- Detailed follow-up after antenatal glucocorticoid treatment in human should focus on hippocampal function, for instance spatial learning.
- Until new treatments to enhance fetal lung development are available obstetricians should be cautious with the use of antenatal glucocorticoids, and multiple courses should be avoided since adverse effects are dose related. Correct identification of 'true' preterm labour essential to reduce unnecessary exposure to glucocorticoids.
- The mechanisms of the mode of action of glucocorticoids structure and function of the lungs needs to be determined to allow the development of more specific agents in the prevention and treatment of respiratory distress syndrome.
- Dose response studies for antenatal and neonatal glucocorticoid treatment should be performed.
- Ideally, neonatal hydrocortisone and dexamethasone should be compared in a randomized controlled trial.
- Careful follow-up of children treated with neonatal glucocorticoids should be performed and should focus on metabolic and cardiovascular diseases in adulthood and on learning and memory late in life.
- Future studies should identify the most beneficial antioxidant regimen and should test this first in rodents and optimizing it in larger animals.

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Chapter 10

Samenvatting in het Nederlands

Samenvatting in het Nederlands

Kinderen die prematuur zijn geboren, hebben na de geboorte vaak ademhalingsproblemen. Sinds het baanbrekende onderzoek van Liggins eind jaren '60, waaruit werd geconcludeerd dat toediening van synthetische corticosteroïden (zgn. stresshormonen) aan zwangeren de foetale longrijping bevordert, zijn deze medicijnen onmisbaar bij de behandeling van kinderen voor en na hun vroegprematuure geboorte.
Aan zwangeren die na 23+5 weken en voor 34 weken amenorrhoe dreigen te bevallen, worden protocollair corticosteroïden toegediend om ernstige longproblemen bij de neonaat, infant respiratory distress (IRD), te voorkomen. Postnataal worden prematuren met ernstige longproblemen (RD-syndroom) behandeld met hoge doses corticosteroïden om de longrijping te bevorderen, het ontstekingsproces in de longen te remmen daarmee ook de ernst van latere, chronische longziekte te verminderen. Corticosteroïden zijn echter uiterst krachtige geneesmiddelen met potentieel ook sterke bijwerkingen waarvoor vrijwel iedere lichaamscel gevoelig is. Zij bevorderen onder andere celdifferentiatie en hebben een remmend effect op celdeling. Behandeling ermee vindt juist plaats in een fase waarin celdelingen van organen, o.a. de hersenen en het hart, volop in gang zijn. Schadelijke effecten van corticosteroïden op de ontwikkeling van verschillende foetale orgaansystemen waren dus te verwachten en zijn dan ook in de literatuur beschreven. Echter, het verklarend mechanisme van de schadelijkheid is grotendeels nog onduidelijk. Meerdere studies suggereren dat oxidatieve stress en verlaagde beschikbaarheid van stikstofoxide (NO) een rol spelen bij de schadelijke effecten van behandeling met corticosteroïden.
Het doel van dit proefschrift was om:

1. enkele schadelijke neveneffecten van ante- en postnatale corticosteroïdbehandeling op te helderen ***(hoofdstuk 2-7)***;
2. te onderzoeken of een postnataal toegediende, uit de kliniek geëxtrapoleerde werkzame dosering van corticosteroïden zorgt voor een toename van oxidatieve stress en een afname van beschikbaarheid van NO in de gebruikte proefdieren ***(hoofdstuk 4-6)***;
3. te onderzoeken of gelijktijdige behandeling met de antioxidanten vitamine C en E, dan wel gelijktijdige behandeling met pravastatine de schadelijke neveneffecten van postnatale corticosteroïden doet verminderen ***(hoofdstuk 4-6)***;
4. te onderzoeken of antioxidantbehandeling bij chronische foetale hypoxie voordelige effecten heeft ***(hoofdstuk 8)***.

Deel I

Antenatale corticosteroïden

In **hoofdstuk 2** hebben we ons gericht op de effecten van een eenmalige antenatale corticosteroïdtoediening op de ontwikkeling van de hippocampus van de muis. In de kliniek worden corticosteroïden tussen een zwangerschapsduur van 23+5 en 34 weken gegeven aan vrouwen

die vroegtijdig dreigen te bevallen. In het muismodel dat wij gebruikten, werd dexamethason toegediend bij een zwangerschapsduur van 15.5 dagen (E15.5), een zwangerschapsduur die qua foetale hippocampale ontwikkeling vergelijkbaar is met die van de menselijke foetus in het derde trimester van de zwangerschap. We onderzochten de effecten van antenatale dexamethasontoediening (0.4 mg/kg) op lichaamsgewicht, het volume van de hippocampus, het aantal neuronen in de CA-zone en de gyrus dentatus (DG) van de hippocampus op verschillende tijdstippen van ontwikkeling: E16, E18, de dag van geboorte (P0), P5, P10, P20 en op de leeftijd van zes maanden (volwassen stadium). Daarnaast bepaalden we op deze tijdstippen het aantal apoptotische cellen (afstervend door geprogrammeerde celdood) in de gehele hippocampus en het aantal delende cellen in de subgranulaire zone van de DG, de plaats waar de meeste celdelingen in de hippocampus plaatsvinden. Lichaamsgewicht, het volume van de hippocampus en het aantal neuronen waren tijdelijk lager na antenatale toediening van dexamethason. Kort na de toediening was het aantal apoptotische cellen toegenomen. Gelijktijdig met de toename van apoptose, was de celdeling in de subgranulaire zone afgenomen. Het aantal delende cellen nam daarna toe op P5 en P10, echter op de volwassen leeftijd van 6 maanden bleek het aantal weer gedaald te zijn.
Concluderend resulteerde antenatale dexamethasonbehandeling in een tijdelijke afname van lichaamsgewicht en hippocampusvolume en in een toename van apoptose in de hippocampus. Tevens vonden we een afname van het aantal delende cellen in de subgranulaire zone van de hippocampus op de (volwassen) leeftijd van zes maanden. Dit vormt een belangrijke bevinding omdat de mate van celdelingen in de subgranulaire zone van de hippocampus op de volwassen leeftijd in eerder gepubliceerde studies gerelateerd bleek aan het vermogen tot leren en de geheugencapaciteit. Onze bevinding zou daarmee kunnen duiden op een verminderde reservecapaciteit c.q. vervroegde veroudering.

In **hoofdstuk 3** hebben we de hippocampus van humane prematuurgeborenen die antenataal met corticosteroïden, meestal betamethason, waren behandeld, vergeleken met de hippocampus van een humane controlegroep prematuurgeboren. In totaal werden 21 premature neonaten geïncludeerd, geboren bij een zwangerschapsduur tussen 24 en 32 weken en overleden tijdens of binnen 4 dagen na de bevalling en bij wie schedelobductie werd verricht. We vonden een significant lagere neuronale densiteit (van grote neuronen en neuronen in het algemeen) in de CA-zone van de hippocampus bij de antenataal met corticosteroïden behandelde foetus ten opzichte van onze controlegroep. We vonden geen verschil in densiteit van kleine neuronen. Daarnaast vonden we geen verschil in myelinisatie, gliose, proliferatie, apoptose of ischemische schade.
De klinische effecten van een verminderde neuronale densiteit zijn moeilijk voorspelbaar, omdat het bijvoorbeeld goed mogelijk is dat deze effecten maar van korte duur zijn en dat plasticiteit (vermogen tot neuraal functieherstel) van hersenen van pasgeborenen een ongewenste afname van neuronen nog kan compenseren. Hiermee komen onze bevindingen, genoemd in hoofdstuk 2 overeen en worden onze overwegingen over een mogelijke indicatie voor verminderde neuronale reserve op latere leeftijd, ondersteund.

Deel II

Neonatale corticosteroïden

In deel II van dit proefschrift ligt de focus op corticosteroïdbehandeling na de geboorte. We testten de hypothese dat het genereren van zuurstofradicalen en de verminderde beschikbaarheid van NO, onderdeel zijn van het mechanisme dat de negatieve bijeffecten van corticosteroïden veroorzaakt.

In **hoofdstuk 4** behandelden we vier groepen pasgeboren ratten; de *eerste groep* met dexamethason in een dosering die vergelijkbaar is met de dosering gebruikt in de kliniek bij prematuren; vanaf de eerste levensdag behandelden we de ratten volgens een afbouwschema 0.5, 0.3, 0.1 mg/kg dag; een *tweede groep* met hetzelfde schema dexamethason en daarbij de antioxidanten vitaminen C en E (respectievelijk, 200 mg/kg/dag en 100 mg/kg/dag) gedurende de eerste zes levensdagen; een *derde groep* met alleen vitamines gedurende de eerste zes dagen; tenslotte een *vierde, controlegroep* met hetzelfde aantal injecties en volume fysiologisch zout.

We keken naar drie moleculaire indicatoren voor oxidatieve stress: expressie van 4-hydroxynonenal (lipidenperoxidatie), heat-shock protein 70 (hsp70) en nitrotyrosine (NT) in de cortex en hippocampus op dag 21 na de geboorte (P21). Dexamethasonbehandeling leidde tot een significante toename in de drie indicatoren voor oxidatieve stress in de cortex ten opzichte van controlepups; er werden echter geen veranderingen gezien van deze eiwitten in de hippocampus. Gecombineerde behandeling van dexamethason met vitamine C en E normaliseerde de expressie van Hsp70 in de cortex; echter, 4-HNE en NT bleven onveranderd verhoogd. Bovendien induceerde de behandeling met alleen vitamine C en E oxidatieve stress in de cortex (Hsp70, 4-HNE, NT: alle verhoogd) en in de hippocampus (NT verhoogd). Daarnaast wogen we op P21 de hersenen en analyseerden we de hersenen histopathologisch en stereologisch op het totale volume en dat van verschillende hersendelen: cortex, hippocampus, diepe-grijze-stof en corpus callosum, verder maten we het gemiddelde neuronenvolume en het aantal neuronen in de cortex en hippocampus. Ten opzichte van de controlegroep induceerde postnatale dexamethasonbehandeling een afname van het totale hersenvolume en neuronen in de hippocampale CA1- en de DG-locatie. Dexamethason in combinatie met vitamine C en E resulteerde in een hoger totaal cerebrum volume en neuronale volumes van de hippocampus ter hoogte van CA1 en de DG. Vitamines alleen hadden geen effect op de genoemde metingen. We stelden vast dat toediening van dexamethason in een vanuit de klinische praktijk geëxtrapoleerde werkzame dosering, bij onze proefdieren leidde tot oxidatieve stress in de hersenen en dat, als deze behandeling werd gecombineerd met vitamine C en E, oxidatieve stress gedeeltelijk verdween. Daaruit zou kunnen worden afgeleid dat ook in de klinische praktijk toevoeging van antioxidanten aan de postnatale corticosteroïdhandeling van prematuren veiliger zou kunnen zijn voor de ontwikkeling van hun hersenen dan de gebruikelijke behandeling met alleen corticosteroïden.

In **hoofdstuk 5 en 6** gebruikten we dezelfde studieopzet, maar in plaats van vitamine C en E gebruikten we pravastatine 10 mg/kg, toegediend gedurende de eerste zes levensdagen. Statines, oftewel de groep HMG-CoA-reductaseremmers, hebben naast cholesterolverlagende

eigenschappen ook effecten op arteriële stijfheid, op trombocytenaggregatie en verbeteren de functie van het endotheel. Deze bijkomende voordelen worden toegeschreven aan een toename van de beschikbaarheid van stikstofoxide (NO). We bepaalden in het plasma op P21 het gehalte aan stikstofoxides (NOx) als maat voor het circulerend NO. NOx was verlaagd in de dexamethasonbehandelde groep. Additionele behandeling met pravastatine normaliseerde het NOx-gehalte. Echter de behandeling met pravastatine alleen, verlaagde eveneens het NOx in plasma. Een verklaring hiervoor zou kunnen zijn dat zuurstofradicalen bij een overschot schade veroorzaken, echter zij hebben ook een rol in fysiologische signaal mechanismen. Door tijdens de behandelperiode NO beschikbaarheid te verhogen, zou het zo kunnen zijn dat pravastatine andere vaatreactiviteit controle mechanismen onderdrukt. Daarnaast is bekend dat het lichaam het verstoorde evenwicht tussen anti-oxidanten en oxidanten zal proberen te herstellen door de productie van meer oxidanten.

Op dag 21 analyseerden we de hersenen (hoofdstuk 5) en ditmaal ook het hart en de bloedvaten (hoofdstuk 6). Dexamethasonbehandeling reduceerde het totale hersenvolume ten opzichte van de controlepups, de hersenen waren proportioneel kleiner met significante verschillen in het corticale en diepe-grijze-stofvolume. Het aantal neuronen in de cortex was lager na dexamethasonbehandeling en ook in de hippocampus was het volume van de neuronen in CA1 en in de DG lager dan in de controlegroep. Daarnaast toonden de met dexamethason behandelde dieren ten opzichte van de controledieren een toename in dichtheid van astrocyten in de witte stof, vlak boven het corpus callosum. Al deze effecten werden niet gevonden in de hersenen van de pups die behandeld waren met de combinatie van dexamethason en pravastatine.

In **hoofdstuk 6** richtten we ons met name op de cardiovasculaire effecten van postnatale dexamethasonbehandeling. We gebruikten hetzelfde behandelprotocol als in hoofdstuk 5 en analyseerden ook in deze studie de pups 21 dagen na de geboorte. Dexamethason-behandeling leidde tot een significante stijging in de gemiddelde arteriële bloeddruk en hartfrequentie, dus hartbelasting (uitgedrukt in Rate Pressure Product) ten opzichte van controlepups. Een toegenomen afterload is zeer waarschijnlijk de oorzaak van dit effect, ten gevolge van hypertensie en toegenomen perifere vasoconstrictie. Gelijktijdige behandeling met pravastatine bij de met dexamethason behandelde pups leidde tot normalisering van het RPP, waarschijnlijk als gevolg van toename van de beschikbaarheid van NO waardoor de perifere vasculaire reactiviteit normaliseert.
Dexamethasonbehandeling leidde tot een verlaagde maximale respons op kaliumchloride en een versterkte gevoeligheid voor de vasoconstrictor phenylephrine, zich uitend in een verhoogde maximale respons. Daarnaast toonde de femoraalarterie van de dexamethasonbehandelde dieren een verminderde gevoeligheid voor de gebruikte endotheel-afhankelijke en endotheel-onafhankelijke vaatverwijders nitroprussidenatrium en metacholine. Gelijktijdige behandeling met pravastatine normaliseerde de perifere reactiviteit voor deze vaattonusbeïnvloedende stoffen. Behandeling met alleen pravastatine verminderde echter de gevoeligheid van de femoraalarterie voor nitroprussidenatrium en metacholine eveneens.

We testten ook het, volgens Langendorff geïsoleerde en doorstroomde rattenhart. Op P21, lieten de geïsoleerde harten van met dexamethason behandelde dieren een lagere basale linkerventrikeldruk en lagere coronaire perfusie zien ten opzichte van controlepups. Gecombineerde behandeling met pravastatine herstelde deze variabelen tot controlewaarden. Na een periode van ischemie vertoonden de harten van de dexamethasonbehandelde dieren een lagere linkerventrikeldruk en lagere hartslag in de eerste tien minuten van het herstel. Ook voor dit effect was gelijktijdige behandeling met pravastatine beschermend. Behandeling van controlepups met alleen pravastatine had geen effect op deze variabelen.
Op P21 lieten pups behandeld met dexamethason alleen, en in combinatie met pravastatine ten opzichte van controlepups een toename zien in de expressie van pro-surfactant proteïne C. Met pravastatine bleef dus het positieve effect op de longen gehandhaafd.
Concluderend ondersteunt hoofdstuk 6 de hypothese dat corticosteroïden de vasculaire functie negatief beïnvloeden door het verlagen van de stikstofoxidebeschikbaarheid, die normaliter de vaatverwijdingsrespons medieert en constrictoragonisten opponeert; tenslotte dat gelijktijdige pravastatinebehandeling de NO-beschikbaarheid herstelt terwijl de pulmonale (pro) surfactantproductie behouden blijft.

In **hoofdstuk 4, 5 en 6** beoordeelden we naast de invloed op hersen- en cardiovasculaire ontwikkeling ook de effecten van postnatale dexamethasonbehandeling op de groei. Dexamethasonbehandeling leidde tot een afname in lichaamsgewicht ten opzichte van controledieren, een effect dat al 24 uur na starten van dexamethasonbehandeling duidelijk werd en nog steeds aanwezig was op de leeftijd van 21 dagen. De groeirestrictie door dexamethason was asymmetrisch, gezien de toename in Ponderal Index, de ratio hersen-:lichaamsgewicht, hersen-:levergewicht, kruin-romplengte:voetgrootte. Gefractioneerde-groeiparameters wordt gebruikt om relatieve groei tussen verschillende groepen in te schatten. Bij controlepups was gefractioneerde groei het grootst gedurende P0-P7, gevolgd door een afname tijdens P9-14. Daarentegen was gefractioneerde groei bij de dexamethasonbehandelde pups juist gereduceerd van P0-7 en toegenomen tijdens P9-P14. Deze laatste toename zou een indicatie kunnen zijn voor 'catch-up growth'.
Postnatale 'catch-up growth', of inhaalgroei, is eerder beschreven bij kinderen na intrauteriene-groeivertraging en wordt beschouwd als een belangrijke risicofactor voor hart- en vaatziekten, hypertensie en insulineresistentie op latere leeftijd.
Indien de bovenstaande resultaten van dierexperimenteel onderzoek ook op de menselijke neonaat van toepassing zijn, moet worden nagedacht over alternatieven voor de tot nu toe in de perinatologie gangbare corticosteroïdbehandeling. Behandeling met de combinatie dexamethason/vitamine C+E gaf geen verandering in lichaams- of hersengewicht t.o.v. de dexamethason-alleen-groep. Bij pups die werden behandeld met de combinatie dexamethason/pravastatine, was de groeionderdrukking minder dan met dexamethason alleen, echter op P21 bleef het lichaamsgewicht toch afwijkend ten opzichte van de controledieren.

In **hoofdstuk *7*** keken we naar de effecten van neonatale dexamethason- en hydrocortison-behandeling op groeipatronen van prematuurgeboren kinderen. We vergeleken longitudinale groeigegevens (lengte, gewicht en hoofdomtrek) van een onbehandelde groep, een met hydrocortison behandelde groep en een met dexamethason behandelde groep. We gebruikten referentiecurves die recent ontwikkeld zijn voor prematuurgeboren kinderen. De studieresultaten vertoonden overeenkomsten met de resultaten van de ratstudie in hoofdstuk 6; met name de groeisnelheid was aangetast. De meest opvallende bevinding was een effect op de gefractioneerde groei van gewicht, lengte en hoofdomtrek. Gefractioneerde groei was over het algemeen gereduceerd in dexamethason- en hydrocortisonbehandelde groepen gedurende de eerste twee maanden, oftewel de behandelperiode, met vervolgens 'catch-up'-groei in de daarop volgende maanden. Daarom suggereren wij in dit hoofdstuk dat deze behandelde kinderen mogelijk een verhoogd gezondheidsrisico hebben en geregeld gevolgd zouden moeten worden ter detectie van metabole en cardiovasculaire aandoeningen. Controles van de hoofdomtrek zijn van belang omdat het groeipatroon hiervan gerelateerd is aan de neurologische en cognitieve ontwikkeling op latere leeftijd, waarbij ook de 'catch-up'-groei tot ongeveer drie maanden gecorrigeerde leeftijd een indicatieve rol speelt voor de uitkomst bij kinderen met een zeer laag geboortegewicht (<1500 g). Dit was juist de periode waarin gefractioneerde groei was afgenomen bij de met corticosteroïden behandelde kinderen en dit zou kunnen wijzen op te verwachten ontwikkelingsstoornissen, hetgeen eerder beschreven is in studies naar de langetermijneffecten van dexamethasonbehandeling. Veranderingen in groeipatronen waren bij de dexamethasongroepkinderen meer uitgesproken dan bij de hydrocortisonbehandelde kinderen. Dit zou een gevolg van structuurverschil in beide medicijnen kunnen zijn, maar er zou ook sprake kunnen zijn van overbehandeling in de dexamethasongroep omdat de behandelingsdosis van dexamethason 2.5 keer potenter was dan de gebruikte hydrocortisondosis.

Deel III

Chronische foetale hypoxie

In **hoofdstuk 8** beschrijven we de resultaten van een voorlopige studie, waarin we een ander mogelijk perspectief voor antioxidantbehandeling testten. Het doel van deze studie was om in zwangere ratten het effect van maternale chronische hypoxie op gedrag en cognitieve functie van hun foetus op latere, volwassen leeftijd te beoordelen en te bezien of de tijdens de hypoxie gegeven xanthine-oxidaseremmer allopurinol neuroprotectieve effecten heeft gehad. Hiertoe werden de zwangere ratten in 4 groepen ingedeeld: de eerste groep, controle groep, verbleef in normoxische atmosfeer met 21% O_2, de tweede groep kreeg een hypoxische ruimte met 13 %O_2, groep drie kreeg 13 %O_2 maar met allopurinolbehandeling (dosering 30 mg/kg) en tenslotte groep vier met 21 %O_2 ook behandeld met allopurinol in voornoemde dosering. Hypoxie en ook allopurinolbehandeling vond plaats vanaf de 6e tot de 20e dag van de zwangerschap. Op de volwassen leeftijd van 3.5 maand werd bij de mannelijke nakomelingen angstgerelateerd gedrag en cognitieve functie geanalyseerd, gebruik makend van verschillende gedragstesten:

de verhoogde-plus-maze-test waarbij angst voor hoogte en open ruimte werd getest en de voorwerp-herkenningstest waarbij 3 en 24 uur na confrontatie met een voorwerp kenmerken van herkenning werd gemeten.
3 uur na voorwerpsconfrontatie was er in de hypoxische groep een significant mindere herkenning van het voorwerp vergeleken met de normoxische groep zonder allopurinol. Dit verschil in herkenning was ook na 24 uur nog zichtbaar, maar statistisch niet meer significant. Deze verminderde voorwerpherkenning was afwezig bij de nakomelingen van de hypoxische groep moeders (13 %O_2) met allopurinolbehandeling. Angstgerelateerd gedrag was niet verschillend tussen de groepen. Allopurinolbehandeling tijdens een normoxische zangerschap had geen waarneembare effecten op objectherkenning en op angstgerelateerd gedrag. Verminderde voorwerpherkenning 3 uur na voorwerpsconfrontatie was ook aanwezig bij de normoxische groep met allopurinol.

Conclusies van dit proefschrift:

- Antenatale toediening van een klinisch relevante dosering dexamethason aan zwangere muizen leidt tot een verminderd aantal celdelingen in de hippocampus van de nakomelingen op de volwassen leeftijd.
- Prematuurgeboren kinderen die antenataal behandeld zijn met corticosteroïden, vertonen een verlaagde neuronale densiteit in de CA-zone van de hippocampus.
- Postnatale dexamethasonbehandeling bij ratten heeft een negatief effect op hersenontwikkeling en cardiovasculaire ontwikkeling.
- Postnatale behandeling met corticosteroïden in combinatie met vitamine C en E of pravastatine zou veiliger kunnen zijn voor het zich ontwikkelende brein en het cardiovasculaire systeem dan behandeling met alleen corticosteroïden.
- Groeipatronen van tevroeggeboren kinderen worden beïnvloed door behandeling met postnatale corticosteroïden (hydrocortison en dexamethason).
- Chronische foetale hypoxie kan de cognitieve functie van het nageslacht beperken; dit negatieve effect kan verminderd worden door maternale allopurinolbehandeling.

Aanbevelingen:

- Follow-up na antenatale corticosteroïdbehandeling zou zich met name moeten richten op de functie van de hippocampus.
- Het is raadzaam om voorzichtig te zijn met het antenataal geven van corticosteroïden. Multipele kuren moeten vermeden worden gezien er sprake is van een dosisgerelateerd effect. Een juiste identificatie van een dreigende partus prematurus is essentieel om het onnodige gebruik van corticosteroïden te reduceren.
- Diepgaand doorgronden van het werkingsmechanisme van corticosteroïden op de onrijpe perinatale longen, zou kunnen bijdragen aan de ontdekking en ontwikkeling van meer specifiekwerkende middelen en methoden ter preventie en behandeling van (dreigende) respiratoire distress bij het kind na vroeggeboorte.
- Het opstellen van dosis-responsstudies voor antenatale en neonatale corticosteroïdbehandeling is essentieel.

- Neonatale behandeling met hydrocortison- en dexamethasonbehandeling zouden met elkaar vergeleken moeten worden in een gerandomiseerde gecontroleerde studie.
- Nauwgezette follow-up van kinderen behandeld met neonatale corticosteroïden zou zich moeten richten op metabole en cardiovasculaire aandoeningen op latere leeftijd, ook op stoornissen van cognitieve functies.
- In toekomstige studies moet de meest gunstig lijkende antioxidantbehandeling verder worden onderzocht en tevens het optimale doseringsschema.

Chapter 11

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List of publications

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Curriculum Vitae

Deodata Tijsseling werd op 1 maart 1983 geboren in Leeuwarden. Zij groeide op in het Friese dorp Stiens en behaalde in 2001 haar gymnasiumdiploma (cum laude) aan het Christelijk Gymnasium Beyers Naudé. In datzelfde jaar startte zij met haar studie geneeskunde aan de Universiteit van Utrecht. Tijdens haar studie verrichtte zij wetenschappelijk onderzoek bij de afdeling neonatologie van het Wilhelmina Kinderziekenhuis. Na deze stage kreeg zij de mogelijkheid als ANIOS te werken op de afdeling neonatologie en te starten met een promotie-onderzoek onder leiding van prof. G.H.A. Visser en prof. F. van Bel. In het voorjaar van 2008 ontving zij verschillende beurzen onder andere van het Internationaliseringsfonds, Stichting de Drie Lichten en de Catharijnestichting om als promovenda onderzoek te kunnen doen aan de Universiteit van Cambridge. Zij ging voor ruim een jaar naar Cambridge en deed onderzoek gesuperviseerd door prof. dr. D.A. Giussani. Van de het onderzoek dat zij verrichtte in Utrecht en Cambridge is dit proefschrift het resultaat. Eind 2009 startte zij als ANIOS bij de verloskunde in het Wilhelmina Kinderziekenhuis. In mei 2011 begon zij met haar opleiding tot gynaecoloog in het Diakonessenhuis te Utrecht (opleider dr. P.C. Scholten). Op dit moment werkt zij als gynaecoloog in opleiding in het Universitair Medisch Centrum te Utrecht (opleider prof. A. Franx).

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Eindelijk bij het allerlaatste gedeelte van dit boekje aangekomen, ongelooflijk, het zit er bijna op!

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