Antenatal corticosteroids and brain development : the use of S100B as an early predictor of brain impairment

Antenatal Corticosteroids and Brain Development:

The use of S100B as an Early Predictor of Brain Impairment

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Contents

Abbreviations

AB	antenatal betamethasone
ANOVA	analysis of variance
BBB	brain-blood barrier
BDNF	brain-derived growth factor
CD	clinically-equivalent dose (corresponding to 12 mg in the woman)
DG	dentate gyrus
DOHaD	developmental origins of adult health and disease
E	embryonic day, e.g. embryonic day 20 – E20
GC	glucocorticoid
HD	half of CD (corresponding to 6 mg in the woman)
HPA	hypothalamic-pituitary-adrenal
IR	immunoreactivity
IVH	intraventricular hemorrhage
MAP2	microtubule-associated protein 2
MWM	Morris water maze
OB	olfactory bulbs
OF	open field test
ORT	object recognition task
P	postnatal day, e.g. postnatal day 2 – P2
RDS	respiratory distress syndrome
SVZ	subventricular zone
^{3}H-Thy	^{3}H-thymidine

Chapter 1

General Introduction

Antenatal betamethasone and brain development: benefits and potential risks

Bruschettini Matteo

Antenatal glucocorticoids: achievements and problems

Infant respiratory distress syndrome (RDS), also known as hyaline membrane disease, is a life-threatening disorder associated with preterm delivery and is caused by developmental insufficiency of surfactant production and structural immaturity in the lungs (Crowley 2003). Among different therapeutic strategies aimed to improve the quality of life of preterm infants, antenatal maternal supplementation with glucocorticoids (GCs) constitutes a gold standard (Pine and Charney 2002; Fraser, Walls et al. 2004). GCs are currently used in about 10 percent of all pregnancies, thus encompassing hundreds of thousands of infants born each year (Matthews, Owen et al. 2002). In the last decades antenatal GC treatment improved pulmonary outcome in high-risk newborns causing a significant decrease in the mortality rate (Roberts and Dalziel 2006).

Though beneficial for lung maturation, this treatment may cause a wide range of side effects in humans, including a reduction in weight and head circumference at birth (Thorp, Jones et al. 2002; Rodriguez-Pinilla, Prieto-Merino et al. 2006), higher total thyroxine levels (Martin, Van Marter et al. 2005), higher GC bioactivity in the cord vein (Kajantie, Raivio et al. 2004) and a suppressed cortisol response to stressors (Davis, Townsend et al. 2004). The hypothalamic-pituitary-adrenal (HPA) axis controls the synthesis and secretion of cortisol. Cortisol in turn inhibits the HPA at different levels, i.e., at the level of the hippocampus, the hypothalamus and the pituitary (Jacobson and Sapolsky 1991). Of note, an impairment in HPA function has been linked to the development of mood disorders like anxiety and depression (Pine and Charney 2002). The effects of antenatal GCs on HPA axis function have been reported in both fetal (Jellyman, Gardner et al. 2004), neonatal (Burlet, Fernette et al. 2005) and adult (Theogaraj, John et al. 2005; Sloboda, Moss et al. 2007) animals. Within the hippocampus, antenatal GCs have been associated with impaired lipid peroxidation (Miller, Chai et al. 2006), increased oxidative damage (Miller, Chai et al. 2006) and neuropeptide Y expression (Velisek 2006), reduced cell proliferation rate (Scheepens, van de Waarenburg et al. 2003) and decreased synaptic density and levels of cytoskeletal proteins (Antonow-Schlorke, Schwab et al. 2003; Colberg,

Antonow-Schlorke et al. 2004). Further, reductions in hippocampal levels of neurotrophic factors like BDNF (Schaaf, Hoetelmans et al. 1997) and S100B (Niu, Hinkle et al. 1997) have been reported.

Little is known about the consequences of antenatal GCs for later life. Antenatal GCs may affect cholinergic neurons and cognitive functions in adulthood (Emgard, Paradisi et al. 2007). However, the effects on cognition- and anxiety- related behavior are controversial (Rayburn, Christensen et al. 1998; Burlet, Fernette et al. 2005; Sloboda, Challis et al. 2005; Velisek 2006).

Antenatal betamethasone (AB) versus dexamethasone

The use of antenatal GCs in the prevention of RDS was first reported in 1968 (Liggins 1968). A randomized controlled trial in 1972 demonstrated that they improved clinical outcome in preterm neonates (Liggins and Howie 1972). In the following decades this treatment became widely used and different dose regimens of synthetic GCs have been adopted. The most widely used compounds are 1) betamethasone which is normally administered at a dose of 12 mg; twice, 24 hours apart and 2) dexamethasone, which is normally administered 4 times at a dose of 6 mg, using 12-hour intervals. In recent years, betamethasone is preferred, because dexamethasone seems to be less effective in promoting lung maturation whilst inducing more serious side effects like intraventricular hemorrhage and retinopathy (Lee, Stoll et al. 2006; Benzon, Chew et al. 2007). Betamethasone is first detectable in the fetal circulation 1 hour after maternal injection, peaking at 3 hours, and decreasing below the limit of detection at 8 hours (Schwab, Coksaygan et al. 2006).

In case delivery does not occur, the use of multiple courses has become more and more common, though the efficacy of this strategy is controversial (Wijnberger, Nikkels et al. 2002; Ogunyemi 2005; Crowther, Haslam et al. 2006). Repeated antenatal GC administration may reduce the need for mechanical ventilation and treatment with surfactant in neonates, but it does not improve composite primary morbidity outcome (Wapner, Sorokin et al. 2006). Actually, more side effects have been described using repeated courses, including an increased risk of RDS (Peltoniemi, Kari et al. 2007).

Thus, it has been proposed to administer the second course of GCs only after a longer period of time, e.g. two weeks after the first course instead of one (Parer 2004). This approach is thought to reduce the detrimental side-effects described following multiple courses of GCs. Yet, other authors have suggested reducing the injection interval from 24 to 12 hours (Haas, McCullough et al. 2005; Haas, McCullough et al. 2006) or decreasing the dose from 12 to 6 mg (Jobe and Soll 2004). Clinical trials need to show whether these new strategies are effective in inducing lung maturation.

A rat model for assessing the neurodevelopmental effect of AB

It is not possible to fully analyze the effects of AB on brain development and functioning in humans. For this purpose, the rat constitutes a suitable model to assess the effects of AB.

In our study dams were injected with either 170 or 85 µg kg^{-1} betamethasone or vehicle-only on day 20 of gestation. With an approximate weight in the pregnant woman of 80 kg, the 170 µg kg^{-1} dose corresponds to a single course of 12 mg betamethasone twice, 24 hours apart. Moreover, we used half of this dose (85 µg kg^{-1}) to investigate whether a lower dose would induce fewer and/or less severe side effects on brain development. As betamethasone plasma half-life is threefold shorter in the rat as compared to the human (Tamvakopoulos, Neugebauer et al. 2002), the second injection occurred at an interval of 8 hours. For more details, see Chapters 3 and 4.

The comparison of the stage of brain development is a major concern with regard to the interpretation of animal studies examining the effects of GCs. A 10- to 14-day-old rat is equivalent to a term human in respect of neural development (Clancy, Darlington et al. 2001). Term rats are therefore equivalent to very preterm human infants, i.e., those who would receive antenatal GCs in utero.

A further concern is the comparison of neural cell cycle times. Ideally, one would like to expose the rat brain to betamethasone for the same number of cell cycles as in the human. Fortuitously, the cell cycle time of the

developing primate brain is 3 to 5 times longer than that of a rodent fetus in the third trimester of pregnancy, which is similar to the difference in betamethasone half-life between primates and rodents (Kornack and Rakic 1998).

The responsivity to corticosteroids differs considerably amongst rat strains. For the purpose of our studies, Fischer 344 rats were used, because these represent a pure breeding inbred strain with low heterogeneity and are known to be reasonably stress responsive (Sternberg, Glowa et al. 1992; Dhabhar, McEwen et al. 1997; Izumi, Washizuka et al. 1997).

Mechanisms

Primary effects of AB involve the activation of corticosteroid receptors. Due to its high numbers of both glucocorticoid (GR) and mineralocorticoid (MR) receptors, the hippocampus, which plays an important role in emotional processing and is involved in the pathophysiology of mood disorders and cognitive problems (Pine and Charney 2002; Martin and Clark 2007), is very sensitive to stress and glucocorticoids (De Kloet, Vreugdenhil et al. 1998). Similarly AB treatment may affect hippocampus development and function.

Possible (permanent) alterations in hippocampal structure and function may be related to altered levels of neurotrophic factors such as S100B, or to an impairment in developmental hippocampal cell proliferation. Below, these and other possible mechanisms that may be involved in inducing the effects of AB are discussed in more detail.

DOHaD (Developmental origins of adult health and disease)

According to the DOHaD (Developmental origins of adult health and disease) concept, the risk of disease in adulthood partly depends upon variations in the prenatal environment, which are often reflected in body weight at birth (Gillman 2005). Low birth weight has been associated with higher risk for disease (Barker 1995). For example, low birth weight has been associated with an increased susceptibility to stress (Nilsson, Nyberg et al. 2001) and depression (Thompson, Syddall et al. 2001; Gale and Martyn 2004) of note,

the relationship between fetal growth and disease risk in later life reflects the sensitivity of growth to adverse antenatal influences, i.e., birth weight is a rough integrated measure of *in utero* events (Gluckman and Hanson 2004).

S100B protein

S100B protein belongs to a multigenic family of calcium-binding proteins (S100 proteins) and has a neurotrophic effect during brain development (Donato 2001). S100B – mainly produced by astrocytes (Pinto, Gottfried et al. 2000) – can be reduced by antenatal insults, e.g. exposure to ethanol (Eriksen, Gillespie et al. 2000), cocaine (Akbari, Whitaker-Azmitia et al. 1994) or maternal stress during gestation (Van den Hove, Steinbusch et al. 2006), thus leading to impaired development of serotonergic neurons (Tajuddin and Druse 1999). Since the half-life of S100B is about 1 hr and it is excreted mainly in urine, S100B concentrations in biological fluids have been shown to be a useful tool to evaluate brain development and damage (Michetti and Gazzolo 2002).

Cell proliferation in the brain

Antenatal GC administration has been shown to be able to suppress the proliferation of hippocampal cells in the rat both *in vitro* (Yu, Lee et al. 2004) and *in vivo* (Scheepens, van de Waarenburg et al. 2003). Though this effect was followed by a catch-up in cell proliferation, it might permanently alter brain structure (Matthews 2000). In our study the ^{3}H-thymidine incorporation method was used to calculate mitotic activity in specific brain regions by measuring the DNA synthetic rate, which is proportional to the rate of cell proliferation (Scheepens, Wassink et al. 2003).

MAP2, Synaptophysin and behavioral tests

Microtubule-associated protein 2 (MAP2) determines stability and arrangement of neuronal microtubules. Loss of MAP2-immunoreactivity (IR) after brain injury correlates with neuronal degeneration (Matesic and Lin 1994) and is associated with age-related impairment of synaptic plasticity, cognition and memory functions. Synaptophysin is a presynaptic marker protein involved in the generation and maintenance of small vesicle

membranes and their interaction with cytoskeletal elements. Decrease in synaptophysin-IR is associated with brain injury and aging, possibly reflecting functional disturbances of synaptic transmission (Martinez, Di Giacomo et al. 1997).

Studies in the sheep and in the baboon have shown that the injection of AB may alter MAP2 and synaptophysin in the fetus and in the neonate (Antonow-Schlorke, Kuhn et al. 2001; Schwab, Antonow-Schlorke et al. 2001; Antonow-Schlorke, Schwab et al. 2003; Colberg, Antonow-Schlorke et al. 2004). However, the long term effects of GCs on MAP2-IR and synaptophysin-IR have not been investigated. Moreover, these morphological changes might underlie the behavioral and learning impairment following GC exposure (Burlet, Fernette et al. 2005; Sloboda, Challis et al. 2005; Velisek 2006).

Aim and outline of the thesis

The focus of this thesis is on the effects of a single course of AB on brain development in human and animal studies. In addition, we investigated the effects of a lower dose regimen.

The aims of this thesis are to investigate the effects of antenatal GCs on:

- fetal growth, brain cell proliferation and S100B levels in the brain, blood and urine;
- cognition- and anxiety- related behavior in the adult offspring;
- MAP2- and synaptophysin-IR in the adult offspring.

Further, we aim to investigate whether lowering the dose of AB might induce less effect on the parameters stated above.

We first focused on the levels of the neurotrophic factor S100B in the urine of infants antenatally treated with betamethasone (Chapter 2). The effects in the rat are presented in the following chapters: on somatic growth and brain cell proliferation (Chapter 3); on S100B concentration in the hippocampus and serum (Chapter 4); on adult cognition- and anxiety-related behavior, and synaptophysin- and MAP2- immunoreactivity (Chapter 5). In Chapter 6, the findings and implications of this thesis are discussed in more detail.

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Chapter 2

Maternal glucocorticoid supplementation and S100B protein concentrations in cord blood and urine of preterm infants

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INTRODUCTION

Maternal glucocorticoid (GC) supplementation is widely used for the prevention of lung immaturity (Crowley 1995; O'Shea, Kothadia et al. 1999), but its possible harmful effects on other organs, including the central nervous system (CNS), are still a matter of debate (Uno, Lohmiller et al. 1990; 1995; Mulder, Derks et al. 1997; Sousa, Madeira et al. 1998; Huang, Beazley et al. 1999; Whitelaw and Thoresen 2000; Vidaeff and Mastrobattista 2001).

S100B, which is present mainly in the nervous system (Heizmann 1999) and has a short half-life (Jonsson, Johnsson et al. 2000), is regarded as a useful marker of brain injury, although at physiologic concentrations it may act as a cytokine with a neurotrophic effect (Michetti, Massaro et al. 1979; Michetti, Massaro et al. 1980; Gazzolo, Vinesi et al. 1998; Gazzolo, Vinesi et al. 1999; Heizmann 1999; Gazzolo, Vinesi et al. 2000; Gazzolo, Bruschettini et al. 2001; Gazzolo, Marinoni et al. 2002; Michetti and Gazzolo 2002). S100B concentrations in cord blood or urine have already been used to monitor brain distress on the fetal/newborn CNS (Gazzolo, Vinesi et al. 2000; Gazzolo, Bruschettini et al. 2001; Gazzolo, Bruschettini et al. 2001; Gazzolo, Bruschettini et al. 2002; Gazzolo, Marinoni et al. 2002; Michetti and Gazzolo 2002).

METHODS

We investigated the possible effects of maternal GC administration on the CNS of newborns by measuring S100B in cord blood and longitudinally in urine. Between April 2000 and June 2002, we measured S100B in the umbilical cord blood and urine of infants, born at our tertiary referral centers for obstetric care, who were admitted to the neonatal intensive care units (NICUs) of our hospitals. From our database we retrieved the 39 infants whose mothers had been treated antenatally with corticosteroids and matched them for gestational age at recording with 39 infants whose mothers had not been treated with steroids (1 control for each patient in the GC group).

Exclusion criteria included multiple pregnancies, fetal/neonatal cardiac or hemolytic diseases, fetal malformations, and chromosomal abnormalities.

The mothers in the GC group had received betamethasone (12 mg intravenously for 2 days) when spontaneous or planned preterm delivery was expected to occur. The main complications in the cases admitted to the study were intrauterine growth retardation (14 of 39 in the GC group; 15 of 39 in the control group), pregnancy-induced hypertension (13 of 39 in the GC group; 14 of 39 in the control group), and preterm delivery not complicated by intrauterine growth retardation, maternal hypertension, or diabetes (12 of 39 in the GC group; 10 of 39 in the control group).

S100B protein was measured at delivery in the umbilical cord blood and subsequently in urine at five predetermined points: at first urination (time 0) and at 24 h (time 1), 48 h (time 2), 72 h (time 3), and 120 h of age (time 4).

On admission to the NICU, all newborns routinely had measurements of red blood cell count, venous blood pH, ion concentrations, plasma glucose, urea, creatinine clearance, osmolality, urinary specific gravity, and arterial blood pressure; all underwent cerebral ultrasound and neurologic examinations. For cases in which GC had been administered, clinical and laboratory tests and cerebral ultrasound scans recorded at the predetermined monitoring time points were reevaluated and compared with those obtained from a control group.

The local Ethics Committees approved the study, and the parents of the infants/patients gave informed and signed consent.

Standard cerebral ultrasound was performed by real-time ultrasound at the same time points as urine sampling, and the grade of intraventricular hemorrhage (IVH) was classified (Papile, Burstein et al. 1978). The neurologic condition of each neonate was classified qualitatively (Prechtl 1974) as normal, suspect, or abnormal. An infant was considered as having an abnormal neurologic condition when hyper- or hypokinesia, hyper- or hypotonia, hemi syndrome, apathy syndrome, or hyperexcitability syndrome

was present. An infant was classified as suspect if isolated symptoms, but no defined syndromes, were present. The same examiner tested all of the infants.

At delivery, the umbilical cord was clamped before any signs of breathing were seen, and blood was drawn from the umbilical vein for S100B measurements. None of the patients who underwent cesarean section experienced uterine contractility before the surgical procedure (Table 1).

Table 1

Characteristics of infants studied.	Controls (n=39)	GC (n=39)
Maternal age,[1] years	27 (2)	26 (3)
Cesarean section, n	15	13
Gestational age at birth,[1] weeks	34 (2)	34 (1)
Birth weight,[1] kg	1.69 (0.56)	1.72 (0.43)
M/F, n	16/13	17/12
Apgar, yes/total		
1st min <7	5/39	4/39
5th min <7	0/39	0/39
RDS, yes/total	16/39	7/39[2]
Neurologic abnormalities, normal/suspect/abnormal	8/14/17	20/19/0[2]
Cerebral ultrasound, normal/IVH	15/24[2]	39/0[2]
Mechanical ventilation support, yes/total	22/39	9/39[2]
Mean NICU stay, days	31	22[2]
Sepsis (within 7 days from birth), yes/total	7/39	5/39

[1] Mean (SD).

[2] *P* <0.05.

Blood and urine samples were centrifuged at 900*g* for 10 min, and the supernatants were stored at -70 ℃ before measurement. S100B was

measured by an immunoluminometric assay (Lia-mat Sangtec 100; AB Sangtec Medical) that specifically measures the ß-subunit, which is known to be predominant in the human brain (Baudier, Glasser et al. 1984; Gazzolo, Bruschettini et al. 2001; Michetti and Gazzolo 2002). The results reported are the means of duplicate measurements. The limit of detection of the assay (B_0 + 3 SD) was 0.02 µg/L, and the intra- and interassay imprecisions (as CVs) were ≤5.5% and ≤10%, respectively, at 0.28–4.2 µg/L.

The data are reported as the mean (SD). Groups were compared by the Kruskal–Wallis one-way ANOVA or by Mann–Whitney *U*-test for non-gaussian-distributed data. The Fisher exact test was used for comparisons of the incidences of neonatal neurologic outcome in patient groups and of acute respiratory distress syndrome (RDS) in patients vs controls.

RESULTS

Fetal and neonatal characteristics are summarized in Table 1. The incidence of RDS, IVH, and neurologic abnormalities, the need for mechanical ventilation support, and NICU hospitalization duration were higher in the infants whose mothers had not received antenatal GC treatment (P <0.05): 20 preterm control infants developed IVH grade I and 4 developed IVH grade II, whereas there were no cases of IVH in the GC group.

S100B measurements in cord blood showed no significant differences between the two groups (P >0.05). In infants born to untreated women, mean blood S100B was significantly higher in group B [brain damage; 3.67 (1.20) µg/L] than in group A [no brain damage; 1.21 (0.24) µg/L] or in infants in the GC group [1.11 (0.24) µg/L; P <0.001]. S100B concentrations in group A and the GC group were not significantly different (P >0.05).

Urine S100B in the GC group showed no temporal trend and ranged from 0.11 (0.08) µg/L at first urination to 0.27 (0.11) µg/L at 120 h. In the untreated group, S100B ranged from 0.59 (0.23) µg/L at first urination to 0.28

(0.19) µg/L at 120 h. S100B concentrations in urine were consistently lower in the GC group (*P* <0.05). Urinary S100B was significantly lower at all predetermined monitoring time points in the GC group than in group B (*P* <0.01) and, notably, group A infants (*P* <0.001; Fig. 1).

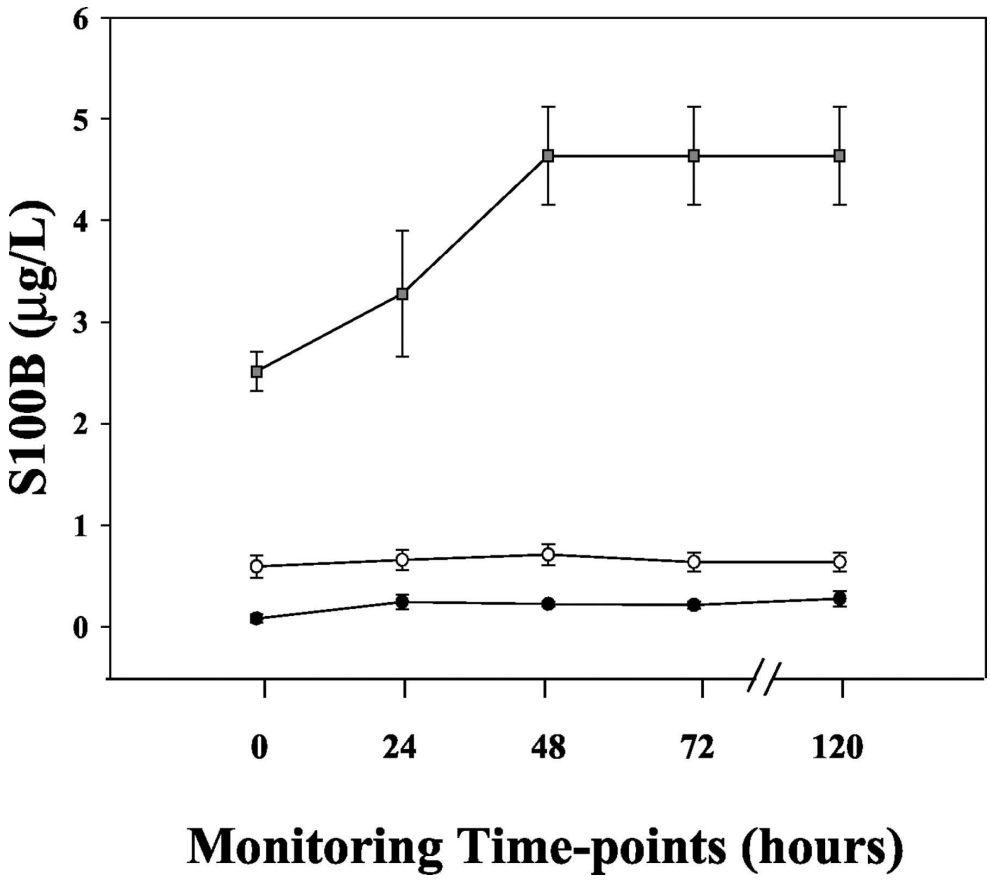

Figure 1. Mean (SD; error bars) urinary S100B concentrations (µg/L) at first urination (time 0) and at 24 h (time 1), 48 h (time 2), 72 h (time 3), and 120 h (time 4) in preterm infants whose mothers were treated antenatally with GC (F) and in controls without (group A) and with (group B) brain damage.
S100B was significantly lower at all monitoring time points in the GC group than in control groups A (P <0.01) and B (P <0.001).

DISCUSSION

The present research shows that a well-established biochemical marker of brain distress, S100B protein, is present in significantly lower concentrations in the urine of newborns whose mothers were treated antenatally with GC than in newborns whose mothers were not treated. Urinary S100B concentrations in infants born to GC-treated mothers were also lower than those in infants without brain damage born to untreated mothers when these were subgrouped according to the occurrence of postnatal brain damage. The results for healthy preterm newborns whose mothers were not treated with GC are in agreement with previous observations of S100B in the urine of healthy preterm infants (Gazzolo, Bruschettini et al. 2001). The high concentrations of urinary S100B observed in brain-damaged newborns whose mothers were not treated with GC offer laboratory support for a previous observation (Gazzolo, Bruschettini et al. 2001).

The presence of S100B in urine has already been demonstrated in healthy newborns, and increased urinary S100B has been shown to be an early indicator of risk for cerebral bleeding (Gazzolo, Bruschettini et al. 2001). Because we found no significant differences in renal function among the three groups studied, the lower S100B in the infants in the GC group is not likely to be attributable to different concentrations of the protein in urine. Furthermore, because S100B is absent from kidney tissue, it is reasonable to suppose that its source in the urine is the CNS (Heizmann 1999; Michetti and Gazzolo 2002). On the other hand, it is possible that at least some S100B may be released from other sites in which it is concentrated, such as adipose tissue (Michetti, Dell'Anna et al. 1983), although data in this setting are lacking.

The longitudinal low S100B concentrations in infants from GC-treated mothers are intriguing. One explanation lies in the lower incidence of neurologic abnormalities in the GC group; these abnormalities almost certainly have a relevant role in the release of the protein into the systemic circulation and the urine. Another explanation may reside in the fact that infants born of GC-treated mothers require fewer intensive care interventions that affect blood–brain barrier permeability (O'Shea, Kothadia et al. 1999) and would thus likely increase the release of S100B into the systemic circulation

(Kapural, Krizanac-Bengez et al. 2002) and finally into the urine. In addition, it should be noted that data on experimental models and in humans support the hypothesis that antenatal GC administration decreases blood–brain barrier permeability, protecting the brain (Ment, Oh et al. 1995; Stonestreet, Petersson et al. 1999).

S100B is regarded as a cytokine with a neurotrophic role at low concentrations and a neurotoxic effect at high concentrations (Haglid, Yang et al. 1997; Heizmann 1999; Jonsson, Johnsson et al. 2000). Our findings of lower S100B concentrations after GC administration open new possibilities for studies aimed at investigating the possible effects of GC on the activity of S100B as a cytokine. Because GCs are known to affect cytokine production in some conditions and GC receptors have been shown to be present in S100B-producing cell types, the possibility that GC action on the CNS is accompanied by a modulation of S100B production and/or release should be taken into consideration (Bohn, O'Banion et al. 1994; Jung-Testas and Baulieu 1998; Elenkov and Chrousos 2002). The present finding of low S100B concentrations after maternal GC treatment appears to be relevant, especially for other perinatal conditions in which GC treatment is used, such as the prevention of bronchopulmonary dysplasia in preterm infants.

Data on S100B measurements in cord blood indicate that GC administration is associated with lower concentrations of the protein in infants not affected by brain injury, consistent with the data regarding urine. Another difference between urinary and cord blood S100B could be related to the possibility that at least a part of the protein present in cord blood has a placental origin, as supported by recent observations (Marinoni, Di Iorio et al. 2002; Wijnberger, Nikkels et al. 2002).

In conclusion, the present findings offer additional data in the debate concerning the effects of antenatal GC administration on the fetal/newborn brain, suggest the use of S100B as a tool to assess the effects of antenatal drug treatment, and offer a clue for future studies on a possible GC-derived modulation of S100B.

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Chapter 3

A single course of antenatal betamethasone reduces neurotrophic factor S100B concentration in the hippocampus and serum in the neonatal rat

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Abstract

The effects of a single course of antenatal betamethasone on S100B protein concentration were investigated in Fisher 344 rats. On day 20 of gestation pregnant rats were injected twice 8 hours apart with either 1) 170 µg kg^{-1} body weight betamethasone ("clinically-equivalent dose", equivalent to 12 mg twice, 24 hours apart in humans), 2) half of this dose (equivalent to 6 mg) or 3) vehicle. We report reference values for S100B protein in the serum and different brain regions in both genders at 1, 2 and 21 days after birth. Interestingly, S100B concentration showed a time-dependent and brain region-specific pattern of expression. At P1, S100B was higher in the serum of males compared to females. In addition, we show that both doses of betamethasone decreased S100B concentration in the serum of males at P1, whereas in the hippocampus it was reduced by the clinically-equivalent dose only. This suggests that lowering the dose of antenatal betamethasone may be less detrimental for brain maturation and therefore we reiterate the need for clinical trials with a low dose regimen.

INTRODUCTION

Antenatal glucocorticoids are widely used in premature infants where they reduce neonatal morbidity and mortality (2000; Pine and Charney 2002; Crowley 2003; Fraser, Walls et al. 2004). However, maternal administration of betamethasone has also been shown to cause a wide range of side effects, including higher thyroxine levels in the first postnatal week (Martin, Van Marter et al. 2005) and neurodevelopmental abnormalities at 2 years of age (Spinillo, Viazzo et al. 2004). In animal models, antenatal glucocorticoids cause a reduction of DNA content (Velazquez and Romano 1987) and cell proliferation rate within the brain (Scheepens, van de Waarenburg et al. 2003), reduced brain growth (Huang, Beazley et al. 1999), loss of synaptic density (Antonow-Schlorke, Schwab et al. 2003), optic nerve hypomyelination (Dunlop, Archer et al. 1997) and an increased susceptibility of cerebellar neurons to oxidative cell death (Fuentes-Pardo, Hernandez-Falcon et al. 1990).

Little is known about the mechanisms responsible for these effects. Glucocorticoids have been shown to be able to reduce neurotrophins levels, e.g. BDNF (Schaaf, Hoetelmans et al. 1997) and S100B. The latter neurotrophic factor is decreased both in the hippocampus, as shown by an in vitro study by Niu and colleagues (1997) and in the urine of infants antenatally treated with a single course of betamethasone (Gazzolo, Kornacka et al. 2003). A lower concentration of the neurotrophic factor S100B might partly explain the detrimental effects of antenatal steroids on brain development. Moreover, S100B levels in biological fluids have been suggested to be a useful and non-invasive tool to monitor brain development and damage (Michetti and Gazzolo 2002). At nanomolar concentrations S100B exerts neurotrophic effects, whereas it is known to be toxic at micromolar concentrations (Donato 2001).

The aims of this investigation were (i) to measure the physiological expression of S100B during development in different brain regions and the serum of the rat; (ii) to investigate the effects of a single course of antenatal betamethasone on S100B concentration by using a clinically-equivalent dose and half of this dose.

METHODS

The animal study was approved by the Animal Ethics Board of the University of Maastricht, The Netherlands.

Animals

Pregnant Fisher 344 dams (Charles River, The Netherlands; pregnancy confirmed by vaginal plug) were delivered to our animal facility on day 14 of gestation (Embryonic day 14, E14). The animals were kept under standard laboratory conditions with 12 hours light/12 hours dark and standard rat chow and water ad libitum. The rats were randomly assigned to an experimental group treated either with a clinically-equivalent dose (CD) of antenatal betamethasone, half of this dose (HD), or vehicle (as specified in the next paragraph).

All the dams delivered on E22, that corresponds to pup age P0 (Postnatal day 0). Pups were analyzed at P1, P2 and P21. For the age between P2 and P21 the pups were all cross-fostered to dams that had given birth on the same day and had received vehicle-only treatment (to prevent a possible betamethasone effect on maternal behavior influencing the results) (Brabham, Phelka et al. 2000). All the litters were kept at 8 pups per dam.

Betamethasone treatment

Dams were injected in the nape of the neck with either 170 or 85 µg kg^{-1} betamethasone (Celestone Chronodose, Schering-Plough, The Netherlands, diluted in its own buffer to a concentration of 230 µg ml^{-1}) or vehicle-only on E20. With an approximate weight in the pregnant woman of 80 kg, the 170 µg kg^{-1} dose represents a clinically-equivalent dose (CD), corresponding to a single course of 12 mg betamethasone twice, 24 hours apart in the woman. Moreover, we used half of this dose (HD, 85 µg kg^{-1}) to investigate whether a lower dose would induce fewer and/or less severe side effects on brain development.

As betamethasone plasma half-life is threefold shorter in the rat compared to the human (Tamvakopoulos, Neugebauer et al. 2002), the second injection occurred at an interval of 8 hours, namely at 9 AM and 5 PM, equivalent to an interval of four half-lives.

An additional crucial difference between humans and rats consists of the timing of brain development. With respect to this issue, a term human infant is equivalent to a 10- to 14-day-old rat (Romijn, Hofman et al. 1991; Clancy, Darlington et al. 2001), therefore betamethasone injection at E20 in the rat occurs when neural development is comparable to very preterm human infants.

S100B measurements

To study the effects of betamethasone on cell proliferation rate within the brain (Bruschettini, Steinbusch et al. 2005) the pups were injected (s.c.) with 5 µCi (g body weight)$^{-1}$ ^{3}H-thymidine (^{3}H-Thy) on their assigned day (either P1, P2, or P21). Following ^{3}H-Thy infusion the pups were kept in a pediatric

incubator at 34℃ and 75% humidity. The pups were s acrificed 1 h later by decapitation and subsequently blood samples were collected from the trunk.

The brains were dissected using the method of Wagner et al. (Wagner, Black et al. 1999) by the same investigator (M.B.) to preserve consistency. The following brain regions were isolated: the olfactory bulbs, the cerebellum, the hippocampus, the sub ventricular zone. The rest of the brain was also taken for analysis. The 5 regions were snap frozen in liquid nitrogen and subsequently stored at -70℃. The dissected brain r egions were then placed in 350 µl (olfactory bulbs and hippocampus) or 1 ml (cerebellum, sub ventricular zone, and rest) pre-cooled lysis buffer containing: 137 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 % NP-40, 10 % glycerol and a protease inhibitor tablet (Roche, The Netherlands). The samples were then homogenized using a Bead Beater (Biospec products, USA) for 3x30 sec, cooling the samples on ice between runs to avoid overheating brain damage.

The S100B protein concentration was measured by use of a commercially available S100B immunoluminometric assay (Liaison, Byk-Sangtec Diagnostica, Germany). This is a two-step sandwich assay based on paramagnetic particles as solid phase and a tracer antibody labelled with an isoluminol derivative. Data reduction is done with a master curve after recalibration with two calibrators.

Since extracellular S100B corresponds to less than 1% of the intracellular content (Tramontina, Conte et al. 2002), our data on S100B content in the brain homogenates reflect essentially the intracellular concentration of the protein. Values are expressed as µg S100B/mg brain weight (brain regions) or µg S100B/L (serum).

Statistics

We used a standardized and randomized block design for these studies whereby each dam had one pup of each sex used at each time point to remove any litter effects (Chapman and Stern 1978). Each experimental group consisted of approximately 8 pups per gender per time point. Data were evaluated with a three-way ANOVA (experimental group x gender x age). Effects were analyzed in more detail with least significant difference (LSD)

post hoc tests (P<0.05). All statistics were carried out using SPSS software version 11.5 (SPSS Inc, USA). Data are presented as means ± S.E.M.

RESULTS

Developmental expression of S100B

Brain region and serum S100B concentrations over time are displayed in Table 1 (vehicle-treated only). An overall effect of age was observed in all the brain regions (P<0.001) except for the olfactory bulbs (Fig. 1). Serum S100B levels in vehicle-treated animals were higher at P21 (1.03 ng/ml) than at P1 (0.83 ng/ml) and P2 (0.69ng/ml) (P<0.01). Post-hoc analysis revealed that this age effect was present in female pups only (P<0.01). In addition, serum S100B levels were lower in female compared to male pups at P1 (-45.5%, P=0.022).

Table 1. Developmental expression of S100B concentrations in the brain and serum in vehicle-treated animals only. An overall effect of age was observed in both genders in the serum (P<0.01) and in all the brain regions (P<0.001) except for the olfactory bulbs. At P1, serum S100B levels were lower in females as compared to males (P=0.022).
Values, expressed as µg S100B/mg brain weight (brain regions) or µg S100B/L (serum), represent means ± SEM; # = female < male.

Region	Gender	P1	P2	P21
Hippocampus	Male	5.07 ± 0.49	9.12 ± 1.65	12.33 ± 0.46
	Female	5.25 ± 0.83	8.17 ± 1.49	12.86 ± 0.94
Cerebellum	Male	6.02 ± 0.28	6.50 ± 0.66	1.43 ± 0.10
	Female	5.19 ± 0.62	6.23 ± 0.33	1.53 ± 0.10
Olfactory bulbs	Male	7.00 ± 3.27	12.93 ± 4.45	8.73 ± 1.75
	Female	8.37 ± 3.77	12.11 ± 3.75	11.62 ± 1.48
Subventricular zone	Male	0.36 ± 0.04	0.63 ±0.02	0.46 ± 0.03
	Female	0.34 ± 0.06	0.48 ± 0.07	0.49 ± 0.05
Rest of the brain	Male	1.84 ± 0.21	1.43 ± 0.27	0.42 ± 0.03
	Female	1.76 ± 0.28	2.14 ± 0.14	0.43 ± 0.03
Whole brain	Male	1.62 ± 0.16	1.98 ± 0.34	1.36 ± 0.10
	Female	1.46 ± 0.28	2.13 ± 0.18	1.59 ± 0.12
Serum	Male	1.14 ± 0.33 #	0.63 ± 0.17	1.15 ± 0.07
	Female	0.52 ± 0.14	0.75 ± 0.19	0.91 ± 0.27

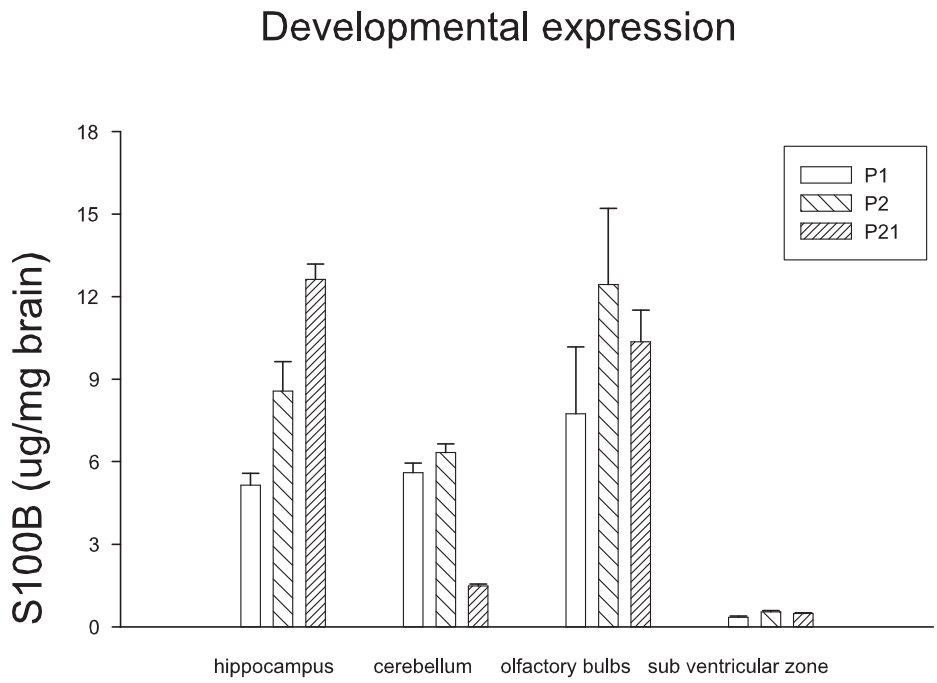

Figure 1. Developmental expression of S100B concentrations within different brain regions, males and females combined. There was an overall effect of age in all the brain regions ($P<0.001$) except for the olfactory bulbs.

Bars, expressed as µg S100B/mg brain weight, represent means ± SEM; † = overtime trend ($P<0.05$).

Effects of antenatal betamethasone on S100B concentration

Fig. 2 shows that CD reduced S100B at P1 in the hippocampus in males (-40.9%, P=0.042), but not in females. In all the other brain regions, S100B was not affected (data not shown). In the serum, S100B was reduced by both CD (-60.0%, P=0.016) and HD (-53.5, P=0.030) in males at P1 (Fig. 3).

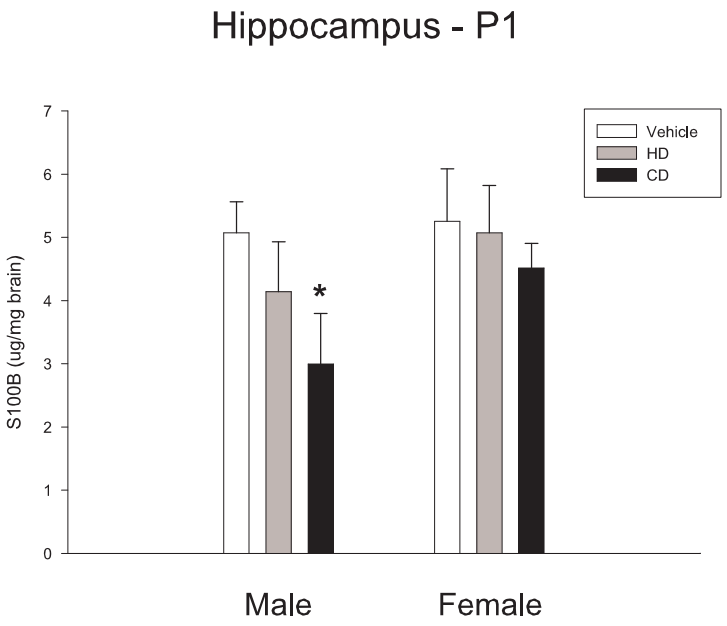

Figure 2. Effects of antenatal betamethasone on hippocampal S100B concentrations at P1. In males, S100B was reduced by CD only (P=0.042). Bars, expressed as µg S100B/mg brain weight, represent means ± SEM; * = P<0.05 compared to Vehicle.

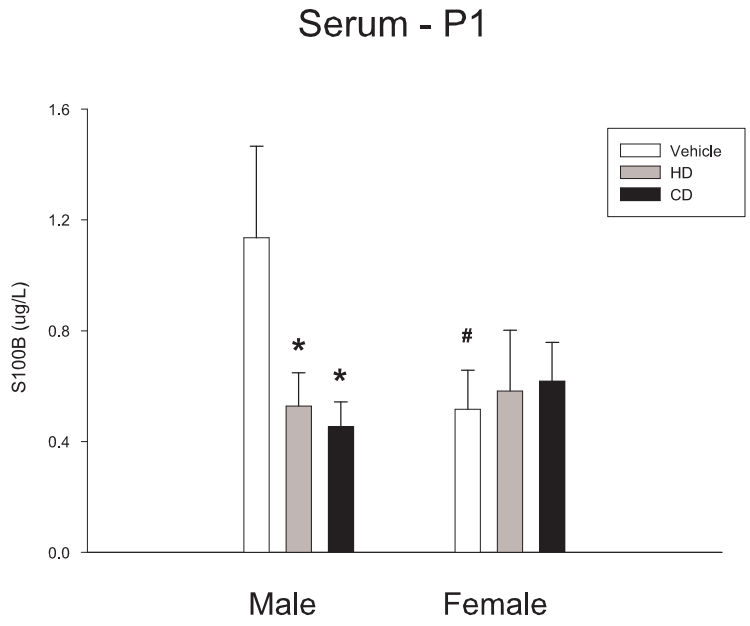

Figure 3. Effects of antenatal betamethasone on serum S100B concentrations at P1. In vehicle-treated pups, S100B levels were lower in females as compared to males (P=0.022). In males, S100B was reduced by both CD (P=0.016) and HD (P=0.030). Bars, expressed as µg S100B/L, represent means ± SEM; * = P<0.05 compared to Vehicle, # = female < male.

DISCUSSION

We report for the first time that a single course of antenatal betamethasone produced a decrease of S100B concentrations in both the hippocampus and serum of male rats. The effect on the hippocampus was dose-dependent, as this region was not affected by the administration of betamethasone at half dose regime.

Developmental expression of S100B

The present study offers reference values for S100B protein in the serum and different brain regions in both genders, showing the developmental expression of S100B at key time points. Neural development in the rat at P1 is equivalent to very preterm human infants, whereas P21 corresponds to full term neonates (Romijn, Hofman et al. 1991; Clancy, Darlington et al. 2001). Our time points therefore cover the whole perinatal period, revealing essential details about brain development. Interestingly, S100B concentration shows a time-dependent and brain region-specific pattern of expression in the rat brain (Pinto, Gottfried et al. 2000; Tramontina, Conte et al. 2002).

Our results confirm that developmental changes occur in S100B expression, i.e., showing the highest concentrations in the cerebellum and sub ventricular zone at P2 and in the hippocampus at P21, whereas there was no trend over time in the olfactory bulbs. Consistently, a developmental increase in S100B concentration has been reported in the human hippocampus, where S100B is already present at 15 weeks' of gestation and increases as from 25 weeks, as was measured immunocytochemically in aborted fetuses (Tiu, Chan et al. 2000).

With respect to S100B circulating concentrations, which have been shown to be a reliable marker for human brain development (Michetti and Gazzolo 2002), we further report that S100B showed the highest levels at P21. Moreover, S100B was higher in males compared to females at P1. Of note, gender differences in S100B blood levels have been reported in investigations in both human infants (Gazzolo, Vinesi et al. 2000) and children (Gazzolo, Michetti et al. 2003). These findings in blood might reflect different patterns of brain development in the two sexes (MacLusky and Naftolin 1981), since the protein is mainly concentrated in the CNS. In addition, gender-

specific differences have been described also in the effect of S100B overexpression on anxiety-related behavior, i.e., transgenic female mice showed hyperactivity in the open field test, whereas behavior is not affected in transgenic males (Gerlai and Roder 1993).

Effects of antenatal betamethasone on S100B concentration

The neurotrophic factor S100B can be reduced by antenatal insults, e.g., exposure to ethanol (Eriksen, Gillespie et al. 2000) or cocaine (Akbari, Whitaker-Azmitia et al. 1994), thus leading to impaired development of serotonergic neurons (Tajuddin and Druse 1999). Interestingly, the stimulation of astrocyte 5-$HT1_A$ receptors might modulate the release of S100B protein (Azmitia, Griffin et al. 1992), that is one of the most potent trophic factors for serotonergic neurons (Azmitia, Dolan et al. 1990; Liu and Lauder 1992). Moreover, acute predator stress has been shown to induce changes in S100B levels in CSF, possibly due to a response to the serotonin effects on astrocytes, although S100B concentration within hippocampus was not affected (Margis, Zanatto et al. 2004).

Steroids are known to reduce neurotrophic factors concentrations, e.g., BDNF in the rat hippocampus (Schaaf, Hoetelmans et al. 1997). In this study, glucocorticoids effects on S100B concentration were investigated in the brain and serum. Two dose regimens of antenatal betamethasone were used: a clinically-equivalent dose (CD) to attempt to replicate the clinical situation of 12 mg twice, 24 hours apart; and half of this dose (HD), equivalent to 6 mg twice, 24 hours apart, according to the idea that it may be an equally effective but less toxic treatment (Jobe and Soll 2004).

We report that CD decreased S100B at P1 in males only, whereas HD did not. In contrast to males, females were not affected by betamethasone at any time point. Of note, this gender difference was found in the same animals regarding cell proliferation within the hippocampus (Bruschettini, Steinbusch et al. 2005). In addition, antenatal betamethasone is known to affect hippocampal mineralocorticoid receptors protein expression more in males than in females (Owen and Matthews 2003). However, it can not be excluded that a decrease in S100B content might have occurred earlier or within a

smaller time window in female compared to males, reflecting gender differences as discussed in the previous paragraph.

Even though at P2 no differences were observed between the different groups, the decreased S100B concentration at P1 might have permanent consequences on brain development. Of note, S100B protein infusion has been recently reported to enhance neurogenesis within the hippocampus in the rat (Kleindienst, McGinn et al. 2005). Thus, a decreased S100B concentration at P1 might imply essential alterations in the cell proliferation within the hippocampus. Moreover, a loss of S100B appears to be detrimental on brain development, as reported in S100B transgenic animal models, where knock-out mice displayed chronic gliosis (Chang, Ariah et al. 2005). In addition, cell damage within the hippocampus due to corticosteroid exposure may induce cognitive deficits (Sousa and Almeida 2002). A lower amount of S100B might be caused by decreased translation or stability of S100B mRNA, or an increased turnover of S100B protein (Eriksen, Gillespie et al. 2000). Interestingly, S100B mRNA levels in hippocampal astrocytes have been reported to be increased 24 hours after corticosteroid exposure and decreased at 96 and 120 hours, whereas S100B protein concentrations were reduced 96 and 120 hours but not increased at 24 hours (Niu, Hinkle et al. 1997). Though the present investigation differs remarkably with the in vitro study by Niu et al, the time interval between corticosteroid treatment and the induced reduction in S100B protein concentration was comparable in both studies.

The region-specific effect of betamethasone on the hippocampus in S100B expression might be explained by the high concentration of glucocorticoid receptors in hippocampal pyramidal neurons, particularly in the CA2 and CA3 regions, where the glucocorticoid receptors concentration is higher than in other cerebral regions (McEwen, Wallach et al. 1974). Consistently, the hippocampus appears to be highly vulnerable to exposure to exogenous glucocorticoids, resulting in acute neural degeneration (Uno, Eisele et al. 1994) and loss of synaptic density (Colberg, Antonow-Schlorke et al. 2004). Moreover, this hippocampal susceptibility was found also in our previous investigation using the same brain homogenates, where antenatal betamethasone affected cell proliferation in this region. Of note, hippocampal

dysfunction has been associated with both cognitive and emotional impairment (see review by Bannerman et al (2004)).

We further report lower S100B levels in the serum of treated male pups at P1. However, the lower circulating levels did not reflect an overall decreased concentration in the CNS, since S100B amount in the whole brain was not affected by betamethasone. This discrepancy might be explained by the specific permeability rates through brain-blood barrier (BBB) in different brain regions (Ilbay, Sahin et al. 2003). In addition, it should be noted that data on experimental models (Stonestreet, Petersson et al. 1999) and in humans (Ment, Oh et al. 1995) support the hypothesis that antenatal glucocorticoids administration decreases BBB permeability. In line with this, the lower circulating S100B levels that we report might be due to an effect of steroids on BBB, independent of any direct brain damage, causing a lower rate of the protein spreading from the brain to the blood. Of note, S100B is highly expressed by perivascular astrocytes and appears in blood seconds after BBB opening (Marchi, Fazio et al. 2003). In this respect, S100B might constitute a useful tool to detect modifications in BBB permeability (Kapural, Krizanac-Bengez et al. 2002; Marchi, Rasmussen et al. 2003; Watson, Shirreffs et al. 2005), which plays an important role in cerebrovascular function.

Conclusion

Hippocampal S100B content was reduced by a clinically-equivalent dose equivalent to 12 mg twice, 24 hours apart of antenatal betamethasone in males, but not by half of this dose. Interestingly, the effect of betamethasone on brain cell proliferation also showed a dose-dependent pattern, as investigated in the same hippocampal homogenates (Bruschettini, Steinbusch et al. 2005).

We speculate that lowering the dose of antenatal steroids may be less detrimental for brain maturation and we therefore reiterate the need for clinical trials with a low dose regimen.

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Chapter 4

Lowering the dose of antenatal steroids: the effects of a single course of betamethasone on somatic growth and brain cell proliferation in the rat

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Abstract

We investigated the effects of a single course of antenatal betamethasone on neonatal somatic and brain development. On day 20 of gestation pregnant rats were injected with either with 170 µg kg^{-1} body weight of betamethasone ("clinically-equivalent dose", equivalent to 12 mg twice, 24 hours apart) or half this dose or vehicle. Pups (8-11 animals per experimental group per timepoint per gender) were analyzed at 1 (P1), 2 and 21 days after birth. We report that betamethasone induced a significant dose-dependent decrease of somatic measurements in both genders. At P1 cell proliferation was affected by the "clinically-equivalent dose" only in the subventricular zone in both genders and in the hippocampus in males. In summary, we show for the first time that a lower dose (equivalent to 6 mg) induces fewer and less severe effects on somatic growth, whereas it does not affect cell proliferation within the brain.

INTRODUCTION

Betamethasone is used in the prevention of respiratory distress syndrome (RDS) in premature babies born between 24 and 34 weeks of gestation. It is known to accelerate maturation of the fetal lungs (Ballard and Ballard 1995) and reduce neonatal morbidity and mortality (2000). However, a single course of antenatal corticosteroids has been shown to decrease birth weight and head circumference at birth (Thorp, Jones et al. 2002) and to affect cortisol response to stressors (Davis, Townsend et al. 2004). Others, however, found no growth restriction following antenatal glucocorticoids (Vermillion, Soper et al. 1999).

In animal models, antenatal glucocorticoids have been shown to cause a wide range of side effects, e.g., reduction of cerebellar DNA content (Velazquez and Romano 1987) and loss of synaptic density in the frontal neocortex, caudate putamen and hippocampus (Colberg, Antonow-Schlorke et al. 2004). In many of these animal studies the doses used had been higher than in the human situation. Recently, our group showed that a single course of antenatal betamethasone (equivalent to 2 injections, 12 hours apart, in the clinic) impaired growth and cell proliferation within the brain of the rat

(Scheepens, van de Waarenburg et al. 2003). In the present study, our primary aim was to investigate the effects of both a standard, clinically-equivalent dose (CD) of antenatal betamethasone (equivalent to 2 injections 24 hours apart) and of half this dose (HD), focusing on somatic growth and neonatal brain development.

METHODS

The animal studies described here were all approved by the Animal Ethics Board of the University of Maastricht, The Netherlands.

Pregnant Fisher 344 dams (Charles River, The Netherlands; pregnancy confirmed by vaginal plug) were delivered to our animal facility on day 14 of gestation (G14). The animals were kept under standard laboratory conditions with 12 hours light/12 hours dark and standard rat chow and water ad libitum. The rats were randomly assigned to an experimental group treated either with a clinically-equivalent dose (CD) of antenatal betamethasone, half this dose (HD), or vehicle (as specified in the next paragraph). Pups were examined at P(ostnatal day)1, P2 and P21. For the age between P2 and P21 the pups were all cross-fostered to dams that had given birth on the same day and had received vehicle-only treatment (to prevent a possible betamethasone effect on maternal behavior influencing the results). All the litters were kept at 8 pups per dam and the pups were kept with the mother until P21, i.e., they were not weaned.

Betamethasone treatment

As mentioned previously, women threatening to deliver preterm are administered 12 mg betamethasone (Celestone Chronodose, Schering-Plough, The Netherlands) twice 24 hours, apart. With an average weight of around 80 kg, this corresponds to 170 µg kg^{-1} betamethasone with a plasma half-life of 6 h in the human. The second injection therefore occurs at an interval of four half-lives. Within the rat, betamethasone has a plasma half-life of 2 h (Tamvakopoulos, Neugebauer et al. 2002). An equivalent dose for a rat would then be two doses of 170 µg kg^{-1}, 8 h apart.

Betamethasone was diluted in its own buffer to a concentration of 230 µg ml^{-1}. The animals were injected in the nape of the neck with a clinically-equivalent dose (CD) of antenatal betamethasone (170 µg kg^{-1}, corresponding to 12 mg in the clinic), half this dose (HD; 85 µg kg^{-1}, corresponding to 6 mg in the clinic) or vehicle-only at 9 AM and 5 PM on G20. All the animals delivered on G22 which was designated as pup age P0. For birth measurements (within 90 mins after delivery) the pups were sexed, weighed and had their crown-tail (C-T) length and head diameter measured by using a digital vernier caliper. All measurements were taken by the same investigator (M.B., blind to the experimental group) to preserve consistency. During this time the dam was never left without any pups and the pups were not separated from their dam for more than 3 min to minimize the stress levels experienced.

Tracer method

We used the ^{3}H-thymidine (^{3}H-Thy) incorporation method to calculate mitotic activity in specific brain regions at 1, 2 and 21 days after birth by measuring the DNA synthetic rate, which is proportional to the rate of cell proliferation.

On their assigned day (P1, P2, or P21) the pups were injected with 5 µCi (g body weight)$^{-1}$ ^{3}H-Thy (25 Ci $mmol^{-1}$, 1 m Ci ml^{-1} in 0.9 % NaCl, Amersham Pharmacia Biotech, The Netherlands) by subcutaneous injection into the nape of the neck. Following ^{3}H-Thy infusion the pups were kept at 34℃ and 75% humidity in a pediatric incubator. Exactly 1 h later the pups were killed by decapitation and the brains were quickly removed and dissected. We isolated 4 brain regions: the olfactory bulbs (OB); the cerebellum; the entire hippocampal formation; and the sub ventricular zone (SVZ) contained within the rostral forebrain. The microdissected regions were then weighed, quickly snap frozen in liquid nitrogen and stored at -70℃. Subsequently the heart, lungs, liver and kidneys were taken and weighed. All dissections were performed by the same investigator (M.B.) to preserve consistency.

The dissected brain regions were then placed in 350 µl (olfactory bulbs and hippocampus) or 1 ml (cerebellum and SVZ) pre-cooled lysis buffer containing: 137 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 % NP-40, 10 % glycerol and a complete protease inhibitor tablet (Roche, The Netherlands).

The samples were then homogenized using a Bead Beater (Biospec products, OK, USA) for 3x30 sec, each time with cooling of the samples on ice between runs.

An aliquot of the homogenate was used to extract all cellular DNA using a standard tri-chloracetic acid precipitation protocol in order to determine the amount of ^{3}H-Thy which was taken up by proliferating cells and incorporated into newly synthesized DNA during the 1 h exposure. Essentially, this measure represents the product of the number of S phase cells within the sample times the DNA synthetic rate of these S phase cells. However, the incorporation of ^{3}H-Thy into DNA depends on the amount of precursor taken up by the tissue. As a consequence, differences in e.g. blood flow between experimental groups might result in differences in ^{3}H-Thy incorporation which does not reflect differences in mitotic activity. For this purpose, another aliquot of the homogenate was used to measure total amount of radioactivity in the tissue fraction.

Homogenized tissue and DNA samples were solubilised in 1 ml Soluene-350 (Packard Instruments, The Netherlands) at 50℃ for 3-24 hours or until the samples were completely dissolved. Afterward 5 ml of Hionic-Flour scintillation cocktail (Packard Instruments, The Netherlands) was added and the samples were read for 20 min on a Wallac WinSpectral 1414 liquid scintillation counter. The appropriate quench curves were produced using tritium standards added to homogenized brain tissue and subsequently used to convert the sample counts per min to disintegrations per min (d.p.m.). The d.p.m. measures were corrected for the wet weight of tissue (mg) and the percentage of ^{3}H-Thy incorporated into DNA relative to the total amount of radioactivity counted in the appropriate tissue of each brain region was calculated.

Statistics

We used a standardized and randomized block design for these studies whereby each dam had one pup of each sex used at each time point to remove any litter effects. Differences in litter size were tested using a one-way analysis of Variance (ANOVA). Mortality was tested using the Fisher exact test. In all other cases, the data were evaluated with a three-way ANOVA

(experimental group x gender x age). Effects were analyzed in more detail with least significant difference (LSD) post hoc tests (P<0.05). All statistics were carried out using SPSS software version 11.5 (SPSS Inc, USA). Data are presented as means ± standard error of the mean (S.E.M.).

RESULTS

Litter size and pre-weaning mortality

No significant differences between groups were observed in litter size (7.6, 7.3 and 9.3 pups/litter for vehicle, HD, and CD, respectively), and pre-weaning mortality (6 pups died: 2 vehicle and 4 CD).

Body weight, head diameter and C-T length at birth

Somatic measurements at birth (P0) are shown in Fig. 1.

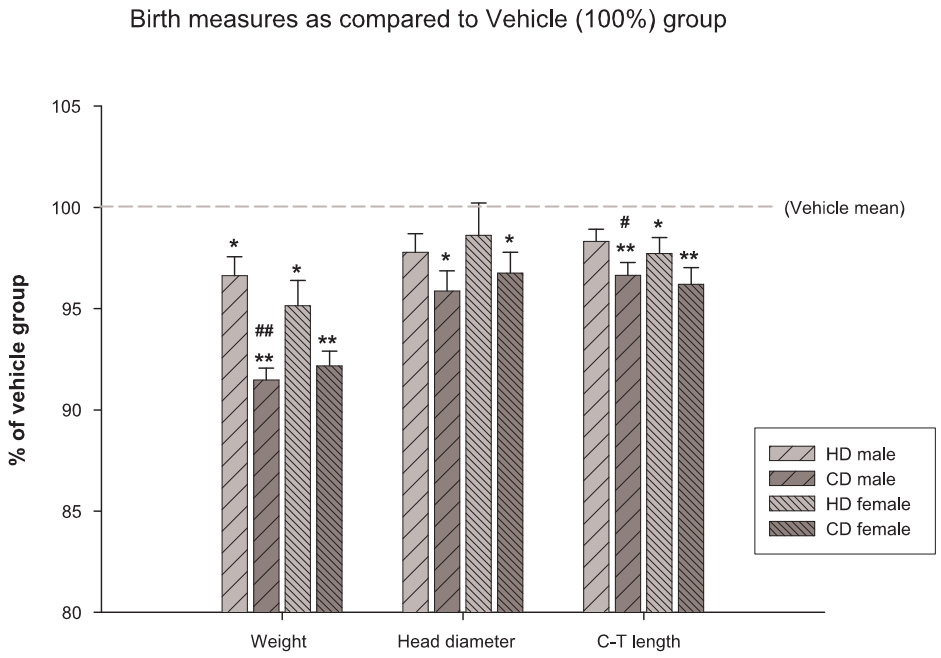

Figure 1. Overview of birth measures - Values are expressed as percentage of the vehicle group and represent Mean + SEM, n= 20 to 34/experimental group/gender, * = P<0.05 compared to Vehicle group, ** = P<0.001 compared to Vehicle group, $^{\#}$ = P<0.05 compared to HD, $^{\#\#}$ = P<0.001 compared to HD; LSD test.

Antenatal betamethasone caused a significant dose-dependent body weight reduction in the CD (-8.0%) and HD (-3.1%) groups. The same effect was found for C-T length (CD: -3.6%, HD: -1.6%).

CD decreased head diameter at birth compared to both vehicle (-3.7%) and HD, whereas head diameter was not significantly reduced by HD. There was a tendency towards a significant effect of HD on head diameter in males ($P = 0.098$).

Female pups were smaller compared to males on all parameters (body weight and C-T length: $P < 0.01$; head diameter $P < 0.05$).

Postnatal somatic growth and relative organ weights

Somatic measures were affected in the treated pups only on P1 (see Table I), with a gender-independent reduction in body weight ($P < 0.01$) and in C-T length ($P = 0.041$), and a tendency for head diameter ($P=0.083$). Post hoc analysis showed that HD did not cause growth retardation, but CD did (body weight: $P=0.028$; C-T length: $P=0.011$; head diameter: $P = 0.032$). C-T length measures were not taken at P21 and gender by experimental group interactions were not found for any somatic measure at any time point.

Overall, relative weights of both the liver and lungs (% of body weight) were reduced by the betamethasone treatment, whereas heart and kidneys were not affected (data not shown).

Brain and brain region weights

CD betamethasone treatment decreased brain weight in both male (-8.7%, $P=0.039$) and female (-6.2%, $P=0.026$) offspring at P1, but not relative weight (brain/body weight ratio).

CD caused an overall decrease in both cerebellum (-11.2%, $P < 0.01$) and hippocampus (-5.4%, $P=0.041$) weight compared to the HD. At P1, SVZ weight was decreased by both doses compared to vehicle in the males (CD: -21.7%, $P < 0.01$; HD: -15.1%, $P=0.016$). At P2, cerebellar weight was reduced only by HD (-17.4%, $P=0.037$), whereas hippocampal weight by CD (-22.3%, $P=0.021$) in the female.

Table 1. Effect of antenatal betamethasone on somatic growth parameters, (mean ± SEM, n= 8 to 11/gender/experimental group/timepoint). Data were evaluated with a three-way ANOVA (experimental group x gender x age). Symbols used for post hoc differences: * = P<0.05 compared to Vehicle, ** = P<0.01 compared to vehicle. CD: clinically-equivalent dose (corresponding to 12 mg twice 24 hours apart in the woman), HD: half CD (corresponding to 6 mg).

Gender and Dose		Parameter	P1	P2	P21
Male	Vehicle	**Body weight (g)**	5.2 ± 0.1	5.8 ± 0.2	26.1 ± 0.6
	HD		4.8 ± 0.1	5.3 ± 0.1	25.8 ± 0.4
	CD		4.6 ± 0.1 **	5.5 ± 0.2	24.6 ± 0.6
Female	Vehicle		4.9 ± 0.1	5.4 ± 0.1	24.6 ± 1.1
	HD		4.7 ± 0.2	5.2 ± 0.2	24.3 ± 0.4
	CD		4.5 ± 0.1 *	5.0 ± 0.2	25.0 ± 0.9
Dose effect			P=0.004	NS	
Gender effect			P=0.059	P=0.04	
Male	Vehicle	**Head diameter (mm)**	10.4 ± 0.2	11.1 ± 0.3	15.6 ± 0.3
	HD		10.2 ± 0.2	10.4 ± 0.2	15.6 ± 0.2
	CD		10.1 ± 0.2	10.6 ± 0.3	15.3 ± 0.2
Female	Vehicle		10.3 ± 0.1	10.6 ± 0.3	15.4 ± 0.3
	HD		10.1 ± 0.3	10.3 ± 0.2	15.2 ± 0.2
	CD		10.3 ± 0.2	10.2 ± 0.3	15.3 ± 0.3
Dose effect			P=0.04	NS	
Gender effect			NS	NS	
Male	Vehicle	**C–T length (mm)**	44.7 ± 0.6	47.6 ± 0.7	
	HD		43.9 ± 0.5	46.0 ± 0.5	
	CD		42.3 ± 0.5 *	46.9 ± 1.0	
Female	Vehicle		43.9 ± 0.5	46.5 ± 0.6	
	HD		43.0 ± 0.6	46.3 ±0.6	
	CD		42.9 ± 1.0	44.8 ± 1.0	
Dose effect			NS	NS	
Gender effect			NS	NS	

Cell proliferation within the brain

At P1, cell proliferation within the hippocampus was higher in CD than in HD in the males (+ 64.2%, P=0.028) (see Fig. 2). In the SVZ, the CD group showed a higher degree of cell proliferation compared to vehicle (+ 55.4%, P=0.007) and in males compared to both HD (+ 82.8%, P=0.011) and vehicle (+ 71.2%, P=0.019). In the rest of the brain, cell proliferation was higher in CD compared to both HD (+38.2%, P=0.002) and vehicle (+34.3%, P=0.002).

At P2, cell proliferation was not significantly changed by betamethasone. At P21, CD increased cell proliferation within the hippocampus compared to vehicle in the females (+4.3%, P=0.050) (data not shown). In the cerebellum, CD increased cell proliferation compared to vehicle (+12.5%, P=0.012) and HD (males only; +11.0%, P=0.014).

Cell proliferation was lower in males in OB (-6.2%, P=0.039), hippocampus (-16.1%, P< 0.01) and SVZ (-12.9%, P< 0.01).

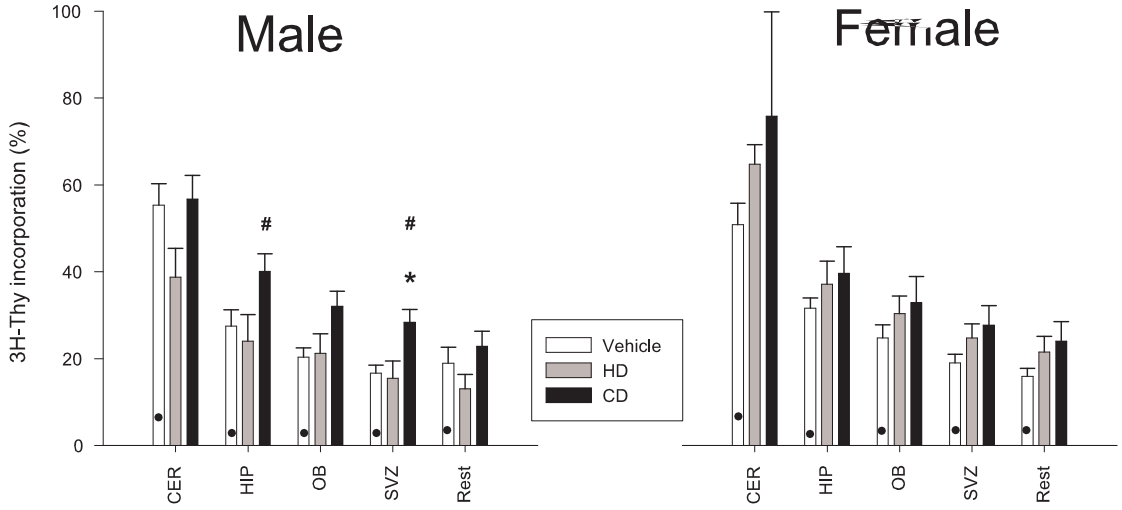

Figure 2. Brain cell proliferation at P1– Graph showing the effect of antenatal betamethasone on postnatal brain cell proliferation (expressed as % ^{3}H-Thy incorporation; see text) in males and females. CD betamethasone treatment caused significant increases in proliferation in HIP and SVZ at P1, whereas HD did not affect cell proliferation in any of the brain regions. For comparison, brain cell proliferation levels at P21 in the vehicle group are depicted by dots.

Values represent Mean + SEM, n= 11 to 17 /experimental group/time; * = P<0.05 compared to Vehicle, $^{\#}$ = P<0.05 compared to HD.

CER: Cerebellum; HIP: Hippocampus; OB: Olfactory bulbs; SVZ: Subventricular zone; Rest: rest of the brain.

COMMENT

The present study shows that a lower dose of antenatal betamethasone results in less severe effects on somatic growth while it does not seem to impair cell proliferation within the neonatal brain.

Recently, our group showed that a single course of betamethasone consisting of two injections 4 h apart in the rat, corresponding to two injections 12 hours apart in the human, induced somatic growth retardation and a reduction of brain cell proliferation (Scheepens, van de Waarenburg et al. 2003). In the present study we chose a 8 h interval, since the Consensus Statement (2000) recommends a 24 hours interval between the two injections. In addition, we used lower doses: a clinically-equivalent dose (CD) to attempt to replicate the clinical situation of 12 mg twice, 24 hours apart; and half this dose (HD). This latter dose regimen (6 mg twice, 24 hours apart) has been recently suggested, since it may be an equally effective but less toxic treatment (Jobe and Soll 2004).

A major concern with regard to the interpretation of animal studies examining the effects of glucocorticoids on neural measures is the comparison of the stage of brain development. Estimates of the rat equivalent age of a term human in respect of neural development have ranged from 7 to 24 days of postnatal age with a general consensus that a 10- to 14-day-old rat is equivalent to a term human (Clancy, Darlington et al. 2001). Term rats are therefore equivalent to very preterm human infants, i.e., those who would receive antenatal glucocorticoids in utero. A further concern is the comparison of neural cell cycle times. Ideally, we would expose the rat brain cells to betamethasone for the same number of cell cycles as occurs in the human. Fortuitously, the cell cycle time of the developing primate (and presumably human) brain is three to five times longer than that of a rodent fetus in the third trimester of pregnancy, which is similar to the difference in betamethasone half-life (Kornack and Rakic 1998).

Somatic growth

The effects of glucocorticoids in pregnancy on growth restriction are not clear in human studies (Vermillion, Soper et al. 1999; Thorp, Jones et al. 2002). We show that a single course of antenatal betamethasone retards somatic

growth. This might be the result of a reduction of circulating levels of growth factors such as growth hormone (GH) and therefore insulin-like growth factor 1 (IGF-1), which are known to stimulate growth (Price, Stiles et al. 1992). Interestingly, at birth HD affected body weight and C-T length less than CD. Moreover, head diameter was not decreased by HD at birth. Of note, the fast growth catch-up observed after betamethasone treatment does not necessarily imply a better outcome. On the contrary, a reduced birth size followed by a fast weight gain has been associated with an additional risk of disease in later life.(Gluckman and Hanson 2004)

In our previous investigation there was no difference in the degree of growth retardation caused by the two doses (except for C-T length in males) (Scheepens, van de Waarenburg et al. 2003). This discrepancy may be due to the lower dose now been used, revealing the presence of a ceiling effect in terms of growth retardation. The longer interval between the two injections may be an alternative explanation, resulting in a lower peak concentration of betamethasone in both the dam and fetus. Obviously, a combination of these two factors, i.e., lower dose and longer injection interval, is also a possible explanation. In line with this finding, HD does not produce a reduction in head diameter, as observed in human neonates following a single course of betamethasone (Thorp, Jones et al. 2002). Of note, a reduced head diameter at birth has been associated with learning problems in school-age children (Stathis, O'Callaghan et al. 1999).

The milder effects found in the present study were also visible in the postnatal growth of the pups, characterized by a prompt catch-up, with a reduction in weight and C-T length only by CD at P1, and no differences after P1. However, a rapid postnatal weight gain may increase the incidence of coronary heart disease in adult life (Gluckman and Hanson 2004).

Taken together, these findings suggest that lowering the dose of antenatal betamethasone may result in fewer and less severe effects on somatic growth.

Cell proliferation within the neonatal brain

It is known that glucocorticoids are essential for maturation in the developing CNS, where they play a pivotal role in the remodeling of axons and dendrites,

and in cell survival (Meyer 1983). Antenatal glucocorticoids administration, however, has been shown to be able to suppress the proliferation of cells derived from the hippocampus of rat embryos (Yu, Lee et al. 2004), possibly permanently altering brain structure (Matthews 2000).

In the present study, the effects of antenatal betamethasone on cell proliferation within the brain are less clear compared to those on somatic growth. Whereas HD did not affect cell proliferation, CD increased cell proliferation in the hippocampus (males only) and SVZ (both genders) at P1. This enhanced cell proliferation might be the rebound of a inhibition of cell proliferation prior to this time point, as observed in our previous investigation, which showed a catch-up in cell proliferation at P2 in all the brain regions studied after an initial decrease observed at P1 (Scheepens, van de Waarenburg et al. 2003). The timing of events, i.e., the inhibition of cell proliferation followed by a catch-up, might have shifted, with the inhibition now occurring before P1 due to the longer injection interval –i.e., lower concentration of betamethasone (see above)– used in the present study.

The altered proliferation rate that we report might involve a downregulation in the expression of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and S100B protein. BDNF is implicated in neuronal proliferation, migration and differentiation, whereas S100B protein promotes proliferation in both neuronal and glial cells (Michetti and Gazzolo 2002; Arcuri, Bianchi et al. 2004). Interestingly, these growth factors have been shown to be reduced by antenatal glucocorticoids in the rat hippocampus (Schaaf, Hoetelmans et al. 1997) and in the urine of human infants (Gazzolo, Kornacka et al. 2003). In particular, S100B protein is reduced in the hippocampus in males only (Bruschettini, Van den Hove et al. 2005), possibly reflecting different patterns of brain development in the two sexes (MacLusky and Naftolin 1981).

As described above regarding somatic growth, the present data suggest that also cell proliferation within the brain is spared by halving the dose of antenatal betamethasone.

Conclusion

We show that a single course of antenatal betamethasone (equivalent to two injections, 24 hours apart) induces somatic growth retardation and affects cell proliferation within the hippocampus and SVZ. Moreover, we show for the first time that a lower dose (equivalent to 6 mg twice, 24 hours apart) induces fewer and less severe effects on somatic growth, whereas it does not affect cell proliferation within the brain. This suggests that lowering the dose of antenatal betamethasone might indeed be effective in minimizing related side-effects, once that clinical trials show its effectiveness for inducing lung maturation. We therefore reiterate the need for randomized clinical trials with a low dose regimen.

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Chapter 5

Cognition- and anxiety-related behavior, synaptophysin- and MAP2-immunoreactivity in the adult rat treated with a single course of antenatal betamethasone

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ABSTRACT

We investigated the effects of a single course of antenatal betamethasone on cognition- and anxiety-related behavior and synaptophysin- and microtubule-associated protein 2 (MAP2)- immunoreactivity in the adult rat hippocampus. On day 20 of gestation pregnant rats were injected with either 1) 170 µg kg^{-1} body weight of betamethasone ("clinically-equivalent dose", equivalent to 12 mg twice, 24 hours apart), 2) half this dose or 3) vehicle. Cognition- and anxiety- related behavior of the offspring was analyzed at an age of 5 months using the Morris water maze, object recognition task and open field test. Subsequently, synaptophysin- and MAP2- immunoreactivity were measured in the hippocampus. We report no detrimental effects of antenatal betamethasone on cognition- and anxiety-related behavior and synaptophysin-immunoreactivity in the adult rat. On the other hand, MAP2-immunoreactivity was decreased by betamethasone in males, suggesting a permanent impairment in the hippocampus. Interestingly, the lower dose appears to have less influence in terms of growth restriction – known to be associated with an increased risk of disease in adulthood. Further research might elucidate whether the betamethasone effect on hippocampal neurons persists later in life and could affect the aging process increasing the risk for neuropathology of the adult.

INTRODUCTION

Antenatal glucocorticoids are widely used to prevent respiratory distress syndrome in case of threat of preterm delivery (2000). Though beneficial for lung maturation, this treatment causes a wide range of side effects in humans, including a reduction in weight and head circumference at birth (Thorp, Jones et al. 2002), and in impairment in both circulating glucocorticoid bioactivity (Kajantie, Raivio et al. 2004) and cortisol response to stressors (Davis, Townsend et al. 2004). Animal studies showed that antenatal glucocorticoids affect the hypothalamic-pituitary-adrenal (HPA) axis (Burlet, Fernette et al. 2005), an impairment of which has been linked to the development of anxiety and mood disorders (Pine and Charney 2002). Further, this treatment has been reported to reduce DNA content (Velazquez and Romano 1987), cell

proliferation rate (Scheepens, van den Hove et al. 2003), synaptic density (Colberg, Antonow-Schlorke et al. 2004) and neurotrophic factor concentrations within the neonatal brain (Bruschettini, van den Hove et al. 2005) and induces neural degeneration within the developing hippocampus (Uno, Lohmiller et al. 1990). However, little is known about the consequences for later life, in particular the effects on cognition- and anxiety- related behavior are unclear (Rayburn, Christensen et al. 1998; Burlet, Fernette et al. 2005).

The presynaptic marker protein synaptophysin is a calcium binding protein that plays an important role in the generation and maintenance of small vesicle membranes and their interaction with cytoskeletal elements. Decrease in the synaptophysin-immunoreactivity (IR) is associated with brain injury and aging, possibly reflecting functional disturbances of synaptic transmission (Martinez, Di Giacomo et al. 1997). The neuronal microtubule-associated protein 2 (MAP2) determines stability and arrangement of neuronal microtubules. Loss of MAP2-IR after brain injury correlates with neuronal degeneration (Matesic and Lin 1994) and is associated with age-related impairment of synaptic plasticity, cognition and memory functions. Interestingly, both synaptophysin-IR and MAP2-IR have been reported to be decreased in the hippocampus shortly after betamethasone injection (Antonow-Schlorke, Kuhn et al. 2001; Schwab, Antonow-Schlorke et al. 2001; Colberg, Antonow-Schlorke et al. 2004), whereas the long term consequences on these parameters are not known. As the hippocampus is involved in learning and memory processes, we aimed to evaluate the effects of a single course of antenatal betamethasone as used in clinical practice on (i) cognition- and anxiety- related behavior and (ii) synaptophysin-IR and MAP2-IR in the adult rat hippocampus.

METHODS

2.1 Animals

The animal study was approved by the Animal Ethics Board of the University of Maastricht, The Netherlands.

Pregnant Fisher 344 dams (Charles River, The Netherlands; pregnancy confirmed by vaginal plug) were delivered to our animal facility on day 14 of gestation (Embryonic day 14, E14). The animals were kept under standard laboratory conditions with 12 hours light/12 hours dark and standard rat chow and water ad libitum.

Dams were injected subcutaneously twice 8 hours apart with either 170 or 85 µg kg^{-1} betamethasone, representing a clinically-equivalent dose (CD, corresponding to a single course of 12 mg betamethasone twice, 24 hours apart in the woman) and half of this dose (HD), respectively, as described in a previous study(Bruschettini, van den Hove et al. 2005). Betamethasone injection occurred at E20 when brain development in the rat is comparable to very preterm human infants (Clancy, Darlington et al. 2001).

All the dams delivered on E22, corresponding to Postnatal day 0 (P0). Pups were labeled with paw cut and cross-fostered to dams that had given birth on the same day and had received vehicle-only treatment (to prevent a possible betamethasone effect on maternal behavior). All the litters were culled at 4 males and 4 females per dam, and the cages cleaned once a week. At P21 the pups were weaned and housed together (2 rats of the same gender and experimental group per cage) up to 5 months of age.

2.2 Behavioral tests

At an age of 5 months, rat performance was assessed in the Morris water maze (MWM; spatial memory), object recognition task (ORT; object memory) and open field test (OF; anxiety).

2.2.1 Morris water maze (MWM).

This test was conducted in a tank (diameter 1.53 m) in which an escape platform (diameter 11 cm) was submerged (1.5 cm) below the surface of the water. The rats started facing the wall of the tank from one of out four different, randomly chosen, starting positions, and were trained to find the invisible platform at a fixed invisible position in the water tank. Abundant spatial cues were provided by the furniture in the room and the presence of the experimenter. A video camera installed above the pool and attached to a PC which registered automatically (EthoVision Color Pro, Noldus, The

Netherlands) the movements of a rat. A trial lasted until a rat had found the platform or until 60 s had elapsed. If a rat did not find the platform within 60 s, it was placed on the platform for 3 s and then removed from the water tank. The rats were given two trials a day for four days and the time between subsequent trials was 10 min. Escape latency was averaged per rat per session of two trials. A probe trial lasting 60 s, during which the platform was removed from the water tank, was given after the last trial to reveal whether the animals had learned the position of the platform. In the probe trial all rats started, facing the wall of the tank, from the position opposite to the position of the removed platform.

2.2.2 Objects Recognition Task (ORT).

The rats were placed in the arena (diameter 83 cm, height 40 cm) facing the transparent segment of the wall, made of polyvinyl chloride. A testing session comprised two trials of 3 minutes each. During the first trial (T1) the apparatus contained two identical objects. After the first exploration period the rat was put back in its home cage for 1 hour and then was put back in the arena for the second trial (T2), but now with two dissimilar objects, a familiar one (the sample) and a new one. The duration of exploring each object in T1 and T2 was recorded manually with a personal computer. Exploration was defined as follows: directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose. Sitting on the object was not considered as exploratory behavior. In order to avoid the presence of olfactory trails, the objects were always thoroughly cleaned. Moreover, each object was available in triplicate so that none of the two objects from the first trial had to be used as the familiar object in the second trial. In addition, all combinations and locations of objects were used in a balanced manner to reduce potential biases due to preferences for particular locations or objects. The light intensity (20 lx) was equal in the different parts of the apparatus. We used four different sets of objects that could not be displaced by a rat. Testing sessions were given on three consecutive days. The basic measures were the total exploration time of both objects during T1 and T2, E1 and E2, respectively. A discrimination index, D2, was calculated as following: D2 = (exploration new object − exploration familiar object) / total exploration time. In addition, each

experimental group was compared to the level expected by chance, i.e. equal amount of time spent on the new and the old object (D2=0). In the last day of test trial, a longer interval (4 hour) between T1 and T2 was used to discriminate cognition differences between experimental groups.

Open Field (OF).

This test was conducted in a square clear Plexiglas box (100 x 100 x 30 cm) with an open top and a dark floor, in a dimly illuminated room. The arena was subdivided into a central zone (64x64 cm), corners (16x16 cm) and walls (16x64 cm). As a rat was placed in the centre of the OF, the position of the animals were registered automatically via a PC (EthoVision Color Pro, Noldus, The Netherlands) through a video camera installed 250 cm above the field for 5 minutes. Testing was carried out for 4 days, consisting of a single 5-min session per day.

2.3 MAP2-IR and synaptophysin-IR

At an age of 6 months rats were anaesthetized by a sodium pentobarbital injection (60 mg/kg, i.p.). Subsequently the brains of the rat were fixated by means of a transcardial perfusion with a flush of tyrode solution (pH 7.4, 4℃) followed by a Somogyi fixation. After dissection of the brain, it was post – fixed in the same fixating for two hours. Thereafter the brain segments were cryoprotected in 20% sucrose in Tris – buffered saline (TBS, pH 7.4) for 48 h and afterwards they were frozen by means of CO_2 and stored at -75° C for further analysis. Sections of 30 µm were cut with the cryostat at levels of the hippocampus (between 1.60 mm and -5.80 mm from bregma) for synaptophysin and MAP2 staining. The serial brain slices of 30 µm were stained with monoclonal antibodies against synaptophysin (mouse anti – synaptophysin, 1: 2000, Chemicon International, Temecula) and MAP2 (monoclonal anti –MAP2 (2a + 2b), Clone AP -20, 1 : 1000, Sigma Chemical Company, St. Louis) by means of the ABC -technique. Before all antibody incubations, washing steps were carried out using subsequently TBS-T (10 min), TBS (10 min) and TBS-T (10 min). Next, the tissue slides were incubated with 5 % Normal Donkey serum (NDS), to minimize the background reactivity. After administration of the primary antibody as mentioned above,

the brain slices were kept overnight at 4℃. Before the secondary biotinylated antibodies were added for an one hour incubation, the slices were repeatedly washed with TBS-T and TBS. Subsequently the brain slices were incubated with a preformed avidin- horseradish peroxidase complex for one hour. Immunostaining was developed by adding DAB solution to the tissue slides which resulted in a brown precipitate. To optimize the color development, the brain slices were washed with Tris-HCl before the administration of the DAB solution. The staining procedure was finished by washing the slices three times with TBS (10 min) and placing the brain slices on glass slides which are allowed to dry for 24 hours. Thereafter the slides are dehydrated in a 70% ethanol line followed by enclosing them with Pertex.

Quantification of the tissue staining was performed morphometrically by the same investigator who was blinded to the experimental protocol. Immunoreactivity (IR) of synaptophysin-IR and MAP2-IR was estimated within different layers of the hippocampus. The synaptophysin staining was examined in the hippocampal subfields CA1 and CA3 of the stratum radiatum and the dentate gyrus (DG), whereas MAP2 –IR was examined in the stratum radiatum (R) and the stratum moleculare (M). For MAP2-IR, slices between -3.14 and -5.80 mm from the bregma were examined and averaged.

For each animal and histochemical staining, multiple areas were measured and the results were averaged. Namely, for the synaptophysin staining, three areas were measured in the CA1 subfield, whereas for the CA3 and the DG two. On the other hand, the MAP2-IR measurements were made in one area, both for the stratum radiatum and the stratum moleculare. The number of animals in each treatment group ranged from 6 to 8 for both the synaptophysin and the MAP2 staining. An Olympus Ax70 microscope equipped with a cooled CCD Olympus Digital video camera F-view was used to detect IR by an image analysis system, slightly modified for detection of grayscale punctae (AnalySIS, Soft Imaging System, Münster, Germany). All measurements were performed on a single focal plane. Shading error correction was performed before measurements to correct for irregularities in illumination of the microscopic fields. Background levels were equalized and the detection threshold was tested and kept at the same level for all samples.

2.4 Statistics

A one-way analysis of variance (ANOVA) was used for somatic measures.

For the acquisition of the MWM, treatment effects were evaluated with a two-factorial (treatment and session) ANOVA with repeated measures over session. For the probe trial, preferences for quadrants were evaluated per group by analyzing with t-statistics whether the time spent in a training quadrant , were the platform was located during training, differed from the chance level (15 s).

The data of the testing sessions of the ORT and OF were aggregated per treatment condition to enhance the reliability of the data. A one-factorial ANOVA was used to analyze the effects between groups.

For MAP2-IR and synaptophysin–IR, treatment effects were analyzed with a one-way ANOVA ($P<0.05$).

All statistics were carried out using SPSS software version 12.0.1 (SPSS Inc, USA). Data are presented as means ± standard error of the mean (S.E.M.).

RESULTS

3.1 Somatic growth

Betamethasone reduced both body weight, head diameter and C-T length at birth ($P<0.001$). This difference in size was not observed anymore at 3 weeks of age. Female pups were always more affected than males on all parameters ($P<0.001$). *Post-hoc* analyses per gender are shown in Figure 1.

3.2.1 Morris water maze (MWM)

Figure 2A depicts the time to find the platform during the acquisition. There was no betamethasone effect except on day 2 ($P=0.044$). All experimental groups showed a reduced escape latency over the different days (time effect; $P<0.001$). In the probe trial, no significant differences were found between groups (Figure 2B). A training quadrant preference was shown in all the males, whereas in the females only for HD ($P<0.05$).

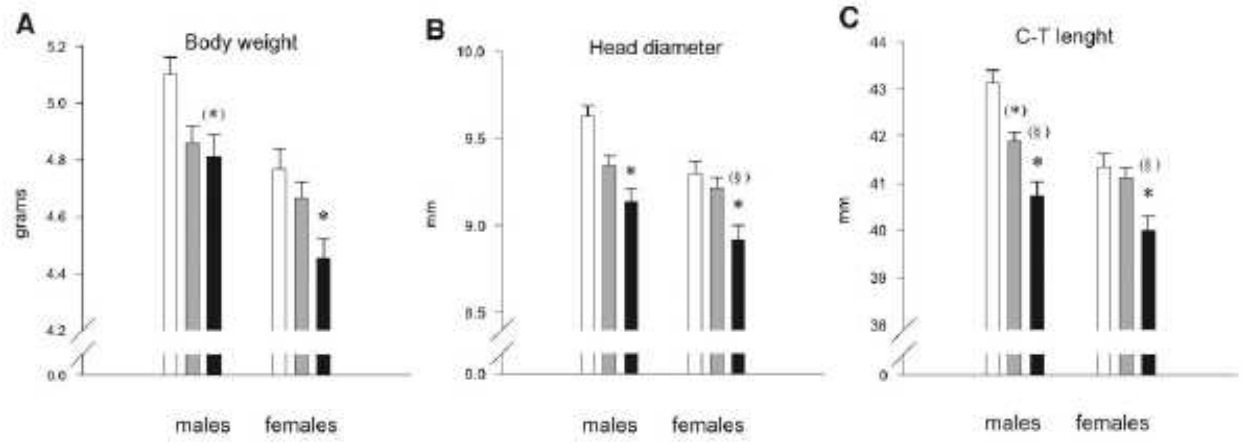

Figure 1. Somatic growth parameters at birth (mean±SEM). Vehicle=white bars, HD=grey, CD=black; n=22-30. Symbols used for Bonferroni *post hoc* differences: (§) = 0.05<P<0.10 compared to HD, (*) = 0.05<P<0.10 compared to Vehicle group, *= P<0.05 compared to Vehicle group.

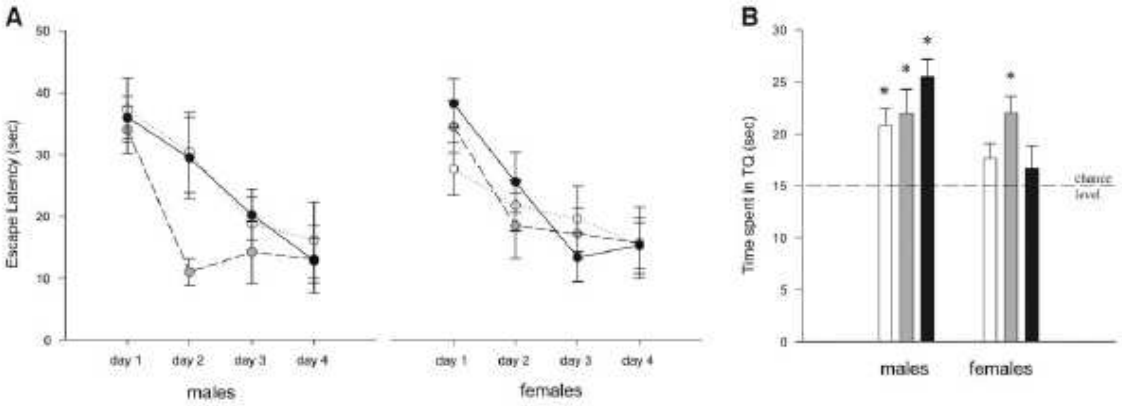

Figure 2. A. Time spent in the Morris Maze during four successive days of training (mean±SEM); Vehicle=dotted lines, HD=dashed, CD=solid; n=10. A treatment effect was found on day 2 combining males and females. **B.** Time spent in the training quadrant in the probe trial; n=10. A dashed line is drawn at 15 s. which indicates the time that animals would have spent in this training quadrant (TQ) at chance level. *=P<0.05 compared to chance level.

3.2.2 Object recognition task (ORT)

There were no significant differences in E1, E2 and D2 between experimental groups (Figure 3). However, by comparing each group with the level expected by chance, only CD resulted in higher D2 in the 4-hours interval ($P<0.05$, males and females combined), whereas there was no treatment effect with the 1-hour interval.

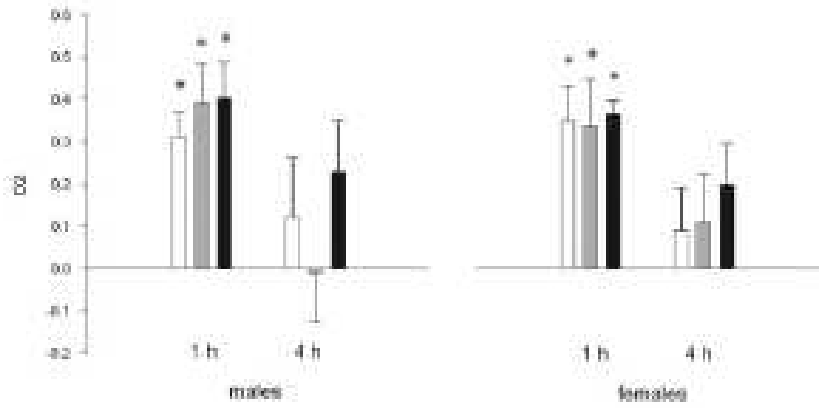

Figure 3. In the object recognition task animals were tested at 1 or 4 hours between the first and the second trial. Values are expressed as mean±SEM; Vehicle=white bars, HD=grey, CD=black; n=10. *=$P<0.05$ compared to chance level, i.e. discrimination index (D2)=0.

3.2.3 Open Field (OF)

The amount of time spent in the different zones of the open filed did not differ between groups (data not shown).

3.3 Synaptophysin-IR and MAP2-IR

Immunohistochemical distribution of synaptophysin-IR revealed an extensive and dense granular pattern in the hippocampus, with big clusters of synaptic vesicles in CA3 region. Antenatal betamethasone caused no significant synaptophysin–IR changes in the three regions examined (data not shown). Males showed higher values than females in both CA3 and DG ($P<0.001$).

Exposure to antenatal betamethasone resulted in a tendency towards a significant decrease of MAP2–IR in both the stratum moleculare ($P= 0.097$) and the stratum radiatum ($P=0.099$) in males (Figure 4). By comparing treated

(CD and HD) versus untreated animals, the loss of MAP2–IR by betamethasone was significant in the stratum moleculare (-18.1%; P=0.031) and border-line significant in the radiatum (-15.4%; P=0.057). Females were not affected by betamethasone treatment. No differences were observed in hippocampus volume.

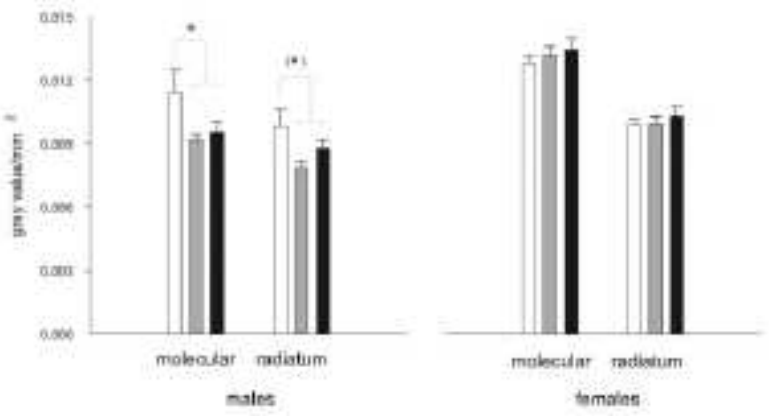

Figure 4. MAP2-IR is expressed as grey value/ μm^2 (mean+SEM); Vehicle=white bars, HD=grey, CD=black; n=10. (*)=0.05<P<0.10 betamethasone-treated compared to vehicle; *=P<0.05 betamethasone-treated compared to vehicle.

DISCUSSION

The present study shows that a single course of antenatal betamethasone impaired fetal growth and MAP2-IR in adult male rats. No significant effect on cognition- and anxiety- related behavior and synaptophysin-IR was observed.

Both body weight, head diameter and crown-tail length were reduced at birth by a single course of betamethasone equivalent to 12 mg twice 24 hours apart (CD) but not by half of this dose (HD). Corticosteroids have been shown to affect fetal growth, probably reducing the circulating levels of growth hormone (GH) and therefore insulin-like growth factor 1 (IGF-1), which are known to stimulate growth (Price, Stiles et al. 1992). In addition, the transplacental transfer of maternal stress hormones, i.e., corticotrophin-releasing factor (CRF) and corticosterone, may affect fetal growth. Moreover, a single course of antenatal betamethasone reduces brain weight in the adult sheep (Moss, Doherty et al. 2005) and head diameter in human neonates

(Thorp, Jones et al. 2002), a finding that has been associated with learning problems in school-age children (Stathis, O'Callaghan et al. 1999). It is well known that reduced birth size is associated with an increased risk of disease in adulthood (Barker 1995). Interestingly, this relationship has been suggested to reflect the sensitivity of fetal growth to adverse antenatal events without implying a causal role of being born small, i.e. birth weight may represent a rough integrated measure of intrauterine processes (Gluckman and Hanson 2004). Even the temporary effect on somatic growth reported in this study might therefore have a significant impact on health, as discussed before in more detail (Gluckman and Hanson 2004; Bruschettini 2006). Of note, in the present study HD appears to have less influence in terms of growth restriction and the implications mentioned above when compared to CD, and a clear dose-dependency in the effect of antenatal betamethasone on fetal growth was previously described by our group using the same animal model and dose regimen (Bruschettini 2006).

To our knowledge, this is the first report of long-term effects of antenatal betamethasone on MAP2-IR and synaptophysin-IR, shown immunohistochemically in the rat hippocampus at 6 months of age, which is comparable to an adult human being.

A glucocorticoid effect on MAP2-IR – an acute effect of which has been previously described in the fetal sheep (Schwab, Antonow-Schlorke et al. 2001) – seems to be permanent, suggesting that antenatal betamethasone induces structural alterations in the hippocampus. In rodents it has been shown that loss of MAP2-IR is associated with neuronal degeneration, which might affect cognition (Matesic and Lin 1994). Of note, Di Stefano and colleagues (Di Stefano, Casoli et al. 2001) have shown a decrease of MAP2–IR during aging in the rat hippocampus. Thus, loss of MAP2-IR in the hippocampus may be associated with age-related impairment of learning and memory.

Glucocorticoids contribute to brain aging by prolonged exposure to excess glucocorticoid, possibly as a result of decreased glucocorticoid receptors and also altered regulation of neuronal turnover in the DG (Nichols, Zieba et al. 2001). Cumulative hippocampal changes by corticosteroids lead to abnormal neuroendocrine functioning, cognitive impairment, and increased

vulnerability to serious life events in people with mood disorders (Brown, Rush et al. 1999). An increase in synaptophysin-IR described with aging suggests a role for synaptophysin in the maintenance of the structural components in the hippocampus (Himeda, Mizuno et al. 2005). The decrease in MAP2-IR was not associated with impairment in learning and memory. However, we cannot exclude that a MAP2-IR decline might result in a cognitive deficit in later life due to aging.

Interestingly, the decrease in MAP2-IR was present in males only. Hippocampal corticosteroid receptor levels are known to be affected in the neonate rat following maternal deprivation (Sutanto, Rosenfeld et al. 1996). Recent evidences suggest that the effects of maternal stress and exogenous glucocorticoids are super imposable (Hougaard, Andersen et al. 2005). Gender-specific stress responses vary depending on developmental age at the time of stress exposure, i.e. male stress responses appear fixed across the lifespan whereas females show a more variable pattern (Bowman 2005). Moreover, recent evidence show that antenatal glucocorticoids induce gender-specific alterations regarding both cognition and hippocampal cholinergic function in adult rats (Kreider, Levin et al. 2005). Steroids affect mineralocorticoid receptor (MR; type I) protein expression within the hippocampus more in males than in females, whereas betamethasone has no effect on glucocorticoid receptor (GR; type II) protein expression (Owen and Matthews 2003). This gender differences in the pattern of MR and GR expression during development may result in different periods of vulnerability to glucocorticoid exposure in fetal life. In the present study female rats might be spared at the age of 6 months by the neuroprotective role exerted by estrogen (Norbury, Cutter et al. 2003). However, a decline in MAP2-IR might occur when estrogens decrease with aging. This awaits further research.

No differences were observed in synaptophysin–IR. Fetal *in vivo* studies concerning the effect of antenatal betamethasone treatment on synaptophysin showed a reduction in IR after administration of betamethasone (Colberg, Antonow-Schlorke et al. 2004). However, in those investigations the animals were analyzed immediately after glucocorticoid exposure. Taken together, antenatal betamethasone appears to induce an

acute synaptophysin-IR decrease (Colberg, Antonow-Schlorke et al. 2004) which does not seem to last as seen in the present work.

Further, betamethasone treatment did not show any negative effects on adult cognition. Interestingly, discrimination in the ORT was increased by CD, whereas HD somewhat improved spatial learning in the MWM, but only in males. In line with this, antenatal betamethasone has been shown to enhance selective memory in the juvenile mouse, continuing into adulthood in males (Rayburn, Christensen et al. 1997) and improves school behavior in 6 years old girls (MacArthur, Howie et al. 1982). On the other hand, perinatal steroid administration in the rat has been associated with impaired spatial memory in a dose-dependent manner, though in this study dexamethasone was injected at P4 (Vicedomini, Nonneman et al. 1986).

In the present study anxiety-related behavior was not affected by betamethasone in the OF. The same finding was found in the mouse (Rayburn, Christensen et al. 1998), whereas postnatal glucocorticoids resulted in a more anxious behavior in the adult rat (Neal, Weidemann et al. 2004). This might be due to the permanent changes induced by perinatal glucocorticoid exposure on the anterior pituitary gland (Burlet, Fernette et al. 2005). In addition, betamethasone effects on anxiety might have been masked by the previous intensive behavioral analysis, i.e. MWM and ORT, with the same strain of rats as been recently suggested (Van den Hove, Blanco et al. 2005).

In summary, we report no detrimental effects of a single course of antenatal betamethasone on cognition- and anxiety- related behavior and synaptophysin-IR in the adult rat. On the other hand, MAP2-IR was decreased in males, suggesting that hippocampus-related cognition may be affected in this gender eventually. Further research might elucidate whether this effect increases with aging and whether both genders are affected in the long run. Finally, HD appears to have less influence in terms of growth restriction when compared to CD, supporting the idea that reducing the dose of antenatal betamethasone might be less harmful (Barker 1995; Gluckman and Hanson 2004), once that clinical trials show its effectiveness for inducing lung maturation.

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Chapter 6

General Discussion

Antenatal betamethasone and brain development: future perspectives

Bruschettini Matteo

Summary of the main findings

The work described in this thesis shows that antenatal betamethasone (AB) may have significant, both short- and long-term, consequences on offspring development.

In the rat model, two dose regimens of a single course of AB were used, i.e., a dose equivalent to that used in clinical practice (12 mg, twice, 24 hours apart) and half of this dose (6 mg).

The major findings reported in this thesis are:

- In the clinic, AB induced a reduction of neurotrophic S100B protein in the urine of human newborns *(Chapter 2)*. Of note, in this clinical study, only the standard dose regimen (12 mg twice 24 hours apart) was used.
- In the rat, AB resulted in significant dose-dependent growth restriction in both genders. Moreover, AB altered neonatal hippocampal S100B concentrations and cell proliferation within the neonatal brain in male offspring *(Chapters 3 and 4)*. Further, in the blood, also the lower dose (6 mg) of AB reduced S100B levels in male pups.
- In adult offspring AB did not affect cognition- and anxiety-related behavior and synaptophysin immunoreactivity (IR) within the hippocampus. However, MAP2-IR was reduced in male rats treated with AB *(Chapter 5)*.
- Overall, males were more affected when compared to females, the latter of which showed no impairment in neonatal hippocampal S100B levels, neonatal brain cell proliferation or MAP2-IR within the adult hippocampus.
- Halving the dose of AB resulted in less growth restriction. Further, no impairment of neonatal hippocampal S100B concentration and cell proliferation within the brain was observed by using the lower dose.

Extrapolation to the human situation

A crucial difference between humans and rats lies within the timing of brain development. Roughly, a term human infant is equivalent to a 2-week-old rat (Romijn, Hofman et al. 1991; Clancy, Darlington et al. 2001), thus betamethasone injection at E20 in the rat occurs when neural development is comparable to very preterm human infants. Subsequently, the pups were analyzed at 1, 2 and 21 days after birth in order to assess the effects a short interval after GC exposure (i.e., 1 and 2 days after birth) and as well at a stage when cell proliferation rate in the brain is as low as in the adult (i.e., 21 days after birth).

A major disadvantage of this animal model is that it does not represent a situation of preterm delivery, since all dams delivered at term. Therefore, it can not be ruled out that this difference with the clinical situation had influenced the neurodevelopmental parameters that we investigated. However, as stated in the Introduction, the principle of injecting rats antenatally with GCs is widely used and accepted and has yielded a lot of valuable information so far (Matthews 2000). Moreover, no animal model mimics the human situation by inducing preterm delivery.

Of note, this study did not aim to evaluate the effects of AB on lung maturation. In this regard, clinical trials are needed to assess the efficacy and safety of new dose regimens of GC.

The effects of AB on somatic growth

As mentioned in the Introduction, the DOHaD (Developmental origins of adult health and disease) concept states that the risk of disease in adulthood partly depends upon variations in the prenatal environment, which are often reflected in body weight at birth (Gillman 2005). We report that the administration of AB resulted in dose-dependent fetal growth restriction (Chapter 4). Consequently, halving the dose of AB, which resulted in a milder effect on birth weight, may have important consequences for adult health and disease.

The effects of AB on S100B protein in the human and in the rat

To our knowledge, we show for the first time that AB reduces S100B concentrations in both humans (in the urine) and rats (in the brain and in the blood).

In our clinical study, S100B levels were measured in the blood and urine of infants exposed twice to 12 mg of betamethasone and a control group that did not received AB. The results showed that AB reduced S100B levels in the urine but not in the blood, the latter of which is probably due to the short half-life of the protein (Michetti and Gazzolo 2002). Urinary S100B was significantly lower in the AB group as compared to controls at each timepoint, i.e., at first urination, at 24, 48, 72 and 120 hours after birth. Since renal function did not differ between the groups and S100B is absent from kidney tissue (Michetti and Gazzolo 2002), it is reasonable to suppose that its source in the urine is the CNS.

In the rat, AB decreased S100B concentrations in the blood and in the hippocampus of males but not in female offspring (see following chapter concerning gender differences). Of note, urine was not analyzed. In support of this finding, it has been reported that dexamethasone is able to reduce S100B protein concentrations in rat hippocampal astrocytes in vitro (Niu, Hinkle et al. 1997).

Interestingly, S100B protein has been shown to enhance neurogenesis within the hippocampus in the rat (Kleindienst, McGinn et al. 2005). Although two days after birth no differences were observed between the different groups, the short-term decrease in S100B concentration might have permanent consequences on brain development, possibly involving essential alterations in cell proliferation rate within the hippocampus. Of note, S100B concentration in the whole brain was not affected by betamethasone. We report that hippocampal S100B was reduced by GC but not by the half dose, suggesting that lowering the dose of AB might induce less severe effects on brain maturation.

In the serum, both doses (12 and 6 mg) of AB reduced S100B levels in male pups. The discrepancy between serum and brain concentrations of S100B might be explained by the specific permeability rates through the brain–blood barrier in different brain regions.

The effects of AB on cell proliferation in the brain

We show that AB alters cell proliferation within the hippocampus and SVZ, whereas a lower dose does not affect cell proliferation within the brain. This increase in cell proliferation might be the rebound of a inhibition of cell proliferation prior to this time point, i.e., a catch-up in cell proliferation after an initial decrease (Scheepens, van de Waarenburg et al. 2003). Moreover, this measure of proliferation is a 'snapshot' of 30-40 minutes worth of proliferation, i.e., the approximate time ^{3}H-thymidine is incorporated into DNA (Boswald, Harasim et al. 1990). Thus, possibly, a decrease in cell proliferation has started within hours of the first betamethasone injection at E20, i.e., 4 days earlier.

Of note, hippocampal neurogenesis in the rat has been shown to be increased by intraventricular S100B infusion (Kleindienst, McGinn et al. 2005). It might be that the initial decrease of cell proliferation within the hippocampus following AB might be related to the lower perinatal levels of S100B.

These data suggest that lowering the dose of antenatal betamethasone might indeed be effective in minimizing related side-effects.

The effects of AB on cognition- and anxiety-related behavior, synaptophysin- and MAP2- immunoreactivity in adulthood

We report that AB reduced MAP2-IR in adult male rats, whereas no significant effect on cognition- and anxiety- related behavior and synaptophysin-IR was observed. It has previously been reported that AB may reduce MAP2-IR measured immediately after AB injection (Schwab, Antonow-Schlorke et al. 2001). However, our study shows for the first time that this effect seems to be permanent, suggesting that AB induces structural alterations in the hippocampus. This effect on MAP2-IR might be associated with the decreased S100B protein concentrations observed in the male pups.

Some of the underlying processes resemble those observed with prenatal stress. For example, a significant decrease in MAP2-IR and synaptophysin-IR has been observed in prenatally stressed adult rats, possibly implying a long-lasting astroglial reaction and reduced dendritic arborization (Barros, Duhalde-Vega et al. 2006). Further, prenatal restraint stress has been reported to reduce S100B protein concentration within the

hippocampus (Van den Hove, Steinbusch et al. 2006). It has been shown that loss of MAP2-IR is associated with neuronal degeneration, which might affect cognition (Matesic and Lin 1994).

In the present study, AB did not impair cognition- and anxiety-related behavior, in contrast with previous studies (Burlet, Fernette et al. 2005; Sloboda, Challis et al. 2005; Velisek 2006). This discrepancy might be due to the different dose of AB used, as few studies have used a clinically relevant dose and only a single course of AB. Further, the choice of different strains might be involved, since Fischer 344 rats are known to be hyperresponsive to stress (Sternberg, Glowa et al. 1992; Izumi, Washizuka et al. 1997). It has been suggested that Fischer 344 rats exposed to GC exposure, when challenged show less reactivity than control rats, as they have already been exposed to stress prenatally (Van den Hove, Blanco et al. 2005). Moreover, the difference between experimental groups might have been masked by the intensive behavioral testing which lasted for 5 weeks.

Gender differences

We report a clear gender-specific effect of AB on S100B levels within the brain and the blood, brain cell proliferation and hippocampal MAP2-IR. Of note, this gender difference appeared to be persistent, since it was observed both in neonatal and adult offspring. The diverse impact on male and female offspring might be related to the different timing of relevant developmental processes over gestation in relation to the timing of the GC exposure (MacLusky and Naftolin 1981).

Elevated plasma adrenocorticotropic hormone concentrations were observed in adult males but not in females (O'Regan, Kenyon et al. 2004). Similarly, maternal GC exposure induced a reduction of plasma corticosterone response to acute stress and hypothalamic noradrenalin in male adult offspring only (Reznikov, Nosenko et al. 1999). In the guinea pig, antenatal GCs increased plasma cortisol in female fetuses, but decreased cortisol levels in male fetuses (Dean and Matthews 1999). Further, they altered the regulation of the pituitary and the adrenal cortex more in male offspring,

resulting in a reduction of anxiety-like behaviour, whilst increasing locomotor activity in females (Owen and Matthews 2007). In addition, antenatal GCs have been shown to impair learning in males, whereas females showed improved cognitive performance (Kreider, Levin et al. 2005). Within the hippocampus, there is a more profound effect of GC on mineralocorticoid receptor expression in males than in females (Owen and Matthews 2003).

Interestingly, AB is known to promote lung maturation to a greater extent in females than in males (Van Marter, Leviton et al. 1990). Thus, others have suggested that a better oxygenation in female offspring might, to some degree, explain the better neurological outcome observed in this gender in clinical practice (Hindmarsh, O'Callaghan et al. 2000). However, our data suggest a limited role for oxygenation in this respect, since all pups were born at term, concomitant with a normal lung development. Nevertheless, this awaits further research.

In our studies, rats were investigated up to the age of 6 months, roughly corresponding to young adulthood, when estrogens still play an important neuroprotective role in females (Norbury, Cutter et al. 2003). More animal research is required to investigate the effects of AB on brain structure and functioning with further ageing.

Conclusion

The present work reports both short- and long-term consequences of AB for neurotrophic factors expression, cell proliferation in the brain and MAP-IR within the hippocampus of male offspring. Of note, a lower dose of AB (equivalent to 6 mg twice 24 hours apart) induced milder effects, supporting the idea that reducing the dose might be less harmful. First however, clinical trials need to show its effectiveness for inducing lung maturation.

Moreover, female rats showed only a reduction of somatic growth, with an extent super imposable to males.

Clinical trials might elucidate whether different, milder, dose regimens could promote proper lung maturation without impairing brain development.

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Summary

Infant respiratory distress syndrome (RDS) is a life-threatening disorder caused by developmental insufficiency of surfactant production and structural immaturity in the lungs of preterm infants. The use of antenatal betamethasone (AB) has resulted in a significant decrease in neonatal mortality and morbidity. However, this treatment strategy may also cause a wide range of side effects in humans, like impairments in somatic growth, brain development and hypothalamic-pituitary-adrenal function. In the present work, the short- and long-term consequences of AB on offspring development were analyzed in human infants and in the rat. Within the rat model, a lower dose regimen of AB was used in order to assess whether lowering the dose might induce milder side effects. The main findings of this thesis are:

In the clinic, AB induced a reduction of neurotrophic S100B protein in the urine of the human newborns *(Chapter 2)*. Of note, in this clinical study, only the standard dose regimen (12 mg twice 24 hours apart) was used.

In the rat, AB resulted in a significant dose-dependent growth restriction in both genders. Moreover, AB reduced hippocampal S100B concentrations and cell proliferation within the brain of neonatal male offspring *(Chapters 3 and 4)*. S100B levels were also reduced in the male blood by the the lower dose (6 mg) of AB.

In adult offspring AB did not affect cognition- and anxiety-related behavior and synaptophysin immunoreactivity (IR) within the hippocampus. However, MAP2-IR was reduced in male adult rats treated with AB *(Chapter 5)*.

Overall, males were more affected as compared to females, the latter of which showed no impairment in neonatal hippocampal S100B levels, brain cell proliferation and MAP2-IR within the adult hippocampus.

Halving the dose of AB resulted in less growth restriction. Further, no impairment of neonatal hippocampal S100B concentration and cell proliferation within the brain was observed by using the lower dose.

In conclusion, the present work confirms that a single course of AB might induce permanent consequences on offspring development. A lower dose of AB (equivalent to 6 mg twice 24 hours apart) induced milder effects, supporting the idea that reducing the dose might be less harmful. First however, clinical trials need to show whether different dose regimens could promote proper lung maturation without impairing brain development.

Curriculum Vitae

Matteo Bruschettini was born on May 7th 1975 in Genoa, located in the Italian Riviera. He graduated at the Liceo Scientifico M.L. King in 1994, and in the same year he started to study Medicine at the University of Genoa. He received his MD degree on July 2000, and in the autumn of that same year he entered the residency in pediatrics at the University of Genoa. Besides his training in the clinics of G. Gaslini Children's Hospital, he actively joined the Perinatal Research Team on S100B protein led by Dr. Diego Gazzolo. In June 2003 he left for Maastricht as a Marie Curie Fellow and then Ph.D. student under the supervision of Professor Harry W.M. Steinbusch, Professor Carlos E. Blanco and Dr. Daniël van den Hove. In August 2005 he moved to Stockholm, where he worked as Guest Researcher in the Neonatal Research Unit at Karolinska University. He is currently working in the Department of Pediatric in Dolo Hospital, nearby Venice (Italy).

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Finally, a big hug to my… big Italian-Estonian family! *Kallis* Olja, you are a special gift in my life; I pray to offer always my best to you.

Publications and Presentations

Journal articles

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Bruschettini M (2007) Antenatal glucocorticoids and CNS development: effects on S100B protein. Invited lecture at G. Garibaldi's Hospital (Catania, Italy)

Bruschettini M (2007) Perinatal glucocorticoids and brain development. Invited lecture at Policlinico Casilino (Rome, Italy)

Bruschettini M (2006) Antenatal glucocorticoids and CNS development. Invited lecture at the International Course in "Recent advances in perinatal medicine" (Erice, Italy)

Bruschettini M, van den Hove DLA, Gazzolo D, Blanco CE, Steinbusch HWM (2006) Cognition- and Anxiety-related behavior, Synaptophysin- and MAP2- IR in the adult rat treated with a single course of antenatal betamethasone. Congress of the European Academy of Pediatrics (Barcelona, Spain). Oral presentation

Bruschettini M (2005) Antenatal glucocorticoid and brain development. Invited lecture at Karolinska Institute (Stockholm, Sweden)

Bruschettini M, van den Hove DLA, Gazzolo D, Steinbusch HWM, Blanco CE (2004) Effects of Antenatal Betamethasone on Somatic Growth and Brain Development. Congress of the Fetal and Neonatal Physiological Society (Castelvecchio Pascoli, Italy). Oral presentation

Posters (most relevant)

Bruschettini M, van den Hove DLA, Gazzolo D, Zimmermann LJI, Steinbusch HWM, Blanco CE (2005) A single course of Antenatal Betamethasone, Growth Retardation, S100B protein, and Cell Proliferation within the Brain and the Lungs. Congress of the American Academy of Pediatrics (Washington DC, USA)

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