PROGRAMMING THE BRAIN

Towards intervention strategies

Sanne Claessens

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Towards intervention strategies
Sanne Claessens
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PREFACE

Synthetic glucocorticoids such as dexamethasone are frequently used to enhance pulmonary development in preterm ventilator-dependent infants. In contrast to the short-term benefit on survival and lung maturation, early glucocorticoid exposure has been shown to adversely affect neurodevelopmental processes. Both human and animals studies have reported acute and long-lasting impairment in response to neonatal dexamethasone treatment. In rodent studies, this treatment is even reported to result in shortening of the lifespan. These findings have led to the question whether the benefits of this treatment outweigh its costs.

Therefore, the objective of the studies described in this thesis is to investigate using an animal model: 1) the short- and long-term consequences of neonatal synthetic glucocorticoid treatment and 2) the possibility to prevent these effects using pharmacological and behavioural intervention strategies.

We report that systemic glucocorticoid treatment acutely affects brain development by suppressing cell proliferation and glial activity. These acute effects on the brain can be partially prevented by central glucocorticoid receptor antagonist pre-treatment. Accordingly, central administration of the antagonist might serve as a protective strategy against the adverse neurodevelopmental effects of dexamethasone treatment.

Although glucocorticoid exposure clearly affects the developmental trajectory, the long-lasting consequences of this treatment were not as detrimental as previously reported. We suggest that daily handling of the neonate, which was an inevitable component of our experimental design and leads to enhanced levels of maternal care towards the offspring, may compensate for the adverse effects of glucocorticoid exposure.

We conclude that the impact of neonatal glucocorticoid exposure highly depends on interactions with other components of the early environment and is therefore susceptible to pharmacological and behavioural intervention strategies.

CHAPTER 1

General Introduction

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It is well documented that early-life experiences are involved in shaping later-life phenotypes. Both human and animal studies have reported the impact of (adverse) early experiences on the development of the stress system, and its consequences for the development of stress-related disorders. The aim of the research described in this thesis is to explore the acute and long-lasting consequences of two distinct types of postnatal experience, varying substantially in nature and severity. The impact of both very subtle differences in maternal environment as well as of exposure to synthetic glucocorticoids during the early postnatal period was investigated. Furthermore, two potential intervention strategies will be introduced to prevent the frequently reported adverse effects of glucocorticoid-induced disruption of normal development and brain maturation.

In this introductory chapter, an overview is given of important concepts for the study of developmental programming. Additionally, several animal models used in experiments described in this thesis will be introduced.

1. DEVELOPMENTAL PROGRAMMING

Early perinatal life represents a critical developmental period. Not only the quality of embryonic environment (1), also early postnatal experiences have long-lasting consequences for emotional and cognitive development and functioning in later-life. Exposure to early adversity such as maternal stress during pregnancy, abuse or exposure to extreme poverty has been shown to increase the vulnerability to develop psychopathology in later-life.

The term 'developmental programming' derives from the concept of 'developmental origin of adult disease' introduced by Barker and colleagues and was based on a vast amount of epidemiological research documenting the relationship between low birth weight and an increased risk of developing metabolic and cardiovascular disorders (2-4). These findings led to what is currently known as the Barker Hypothesis (5). This concept has been since extended and is now frequently studied in the context of the hypothalamic-pituitary-adrenal (HPA) axis (6-10). Programming of the stress system is achieved through the actions of environmental cues acting at a specific time during development, resulting in permanent alterations in the functioning of the HPA axis (6-10).

Both preclinical and clinical evidence suggests that this phenomenon has relevance for the etiology of mental disorders triggered by stressful life events, including depression and post-traumatic stress disorder (9-12).

2. DISRUPTION OF NORMAL DEVELOPMENT

2.1 Human studies

Because of ethical considerations, the direct impact of stress on development cannot be investigated in humans. Therefore progress in this field relies on retrospective reports and correlational studies. There are however some *experiments of nature*

in which the impact of prenatal (intrauterine growth restriction, low birth weight) and postnatal (low socioeconomic status, maltreatment) adversity can be studied.

A series of studies have convincingly shown that exposure to early adverse events, such as childhood abuse, results in an increased risk to develop psychiatric dysfunctions (11-14). A dose-response relationship has been described between the number of experienced childhood adversities and mental health score in later-life (i.e. probability of lifetime depressive disorders) (15, 16). Besides these severe forms of adversity such as emotional neglect and abuse, there is also evidence that milder forms of adversity are associated with increased risk for stress related-pathologies. For instance, early-life socioeconomic disadvantage (17) but also subtle differences in parenting style (18-20) appear to affect health status of the individual in later-life. Therefore, there is no doubt that early adversity plays a crucial role in programming the development of a range of physical and psychiatric disorders which is likely to be mediated (at least partially) via the effects of early adversity on the functioning of the HPA axis. Several studies have shown the association between early-life adversity and enduring sensitization of the responsiveness of the HPA axis in humans. For instance, alterations in basal as well as stress-induced HPA axis activity at different life stages have been reported in human subjects exposed to adversity in early-life (13, 14, 21, 22).

2.2 Animal studies

In contrast to human studies, animal studies allow the development of experimental models where individuals are submitted to acute or chronic adversity and the resulting outcome on brain and behaviour can be investigated. Experimental early-life manipulations can be largely subdivided in prenatal and postnatal manipulations. Prenatal manipulations involve stress during pregnancy, maternal synthetic glucocorticoid exposure, or nutrient restriction. For a review on the impact of prenatal manipulations, we refer to previous literature (23-26).

Postnatal manipulations frequently involve manipulating or depriving the infant from maternal behaviour.

2.2.1 Handling

In the 1950's, it was discovered that exposing rat pups to daily handling sessions, which consisted of brief periods of separation from the dam (< 15 min) between postnatal day (pnd) 1 and 21, had a surprising and unexpected outcome (27). Levine, and others, found that handling induced long-lasting changes in adult phenotype such as HPA axis *hypo*-responsiveness (28-30), reduced emotionality (29), and increased cognitive performance (31) when compared to rats raised in undisturbed laboratory conditions, i.e. non handled. However, the use of such 'undisturbed' control groups was recognized to be problematic later; see reviews (32-34). Because the handling procedure was considered at that time to be a stressful experience, these findings challenged the dominant theory stating that early-life stress invariably contributes to the development of 'emotional instability'. Instead, the findings from Levine demonstrated that, in some instances (e.g. via handling), exposure to 'moderate stress' in early-life appeared to be beneficial for

the infant by promoting a greater ability of the organism to adapt to psychological and physiological stressors in adulthood (27). This same principle also serves as the basis for the stress inoculation-induced resilience theory developed several years later (35-39).

→ *In chapter 5 of this thesis the beneficial effects of neonatal handling will be used as a potential rescue strategy to compensate for the adverse effects of neonatal synthetic glucocorticoid exposure.*

2.2.2 Maternal separation

Over the years new paradigms were introduced in an attempt to also study the mechanisms underlying developmental programming following exposure to more 'adverse' experiences (40). Maternal separation consists of prolonged periods of maternal absence ranging from 1h to 24h. The reported effects of maternal separation appear to be more controversial compared to the effects of handling, in part because of the substantial variety in different experimental procedures across different laboratories in terms of duration, frequency, age of onset of the separation, gender and the choice of control group (41-43). Nevertheless, maternal separation appeared to 'program' the functioning of the HPA axis. As expected, this manipulation was reported to yield a more severe outcome, opposing the effects of handling, including HPA *hyper*-responsiveness following stress (40), increased emotionality (44), and impaired cognitive performance (28). For an extensive review of the consequences of postnatal manipulations, see: (28, 45).

3. MATERNAL MEDIATION HYPOTHESIS

The use of the handling model in rodents raised an important question: how can short episodes of maternal absence result in such profound and enduring effects on adult stress-phenotype? The 'maternal mediation hypothesis' was proposed for the fist time as part of the mechanism underlying the lasting effects of handling by Smotherman and Bell (46). This theory postulates that the outcome of postnatal manipulations (such as handling and maternal separation) is mediated by changes in maternal behaviour directed towards the offspring upon reunion after a given period of mother-infant separation (47). It was observed that brief (15 min) episodes of handling resulted in increased levels of maternal care upon reunion between mother and offspring, sometimes reported to remain higher throughout the entire day (48). Longer periods (4 h) of maternal separation yielded an increase in active maternal care only directly after reunion of the dam with the pups but not at any other time point, leading overall to differences in the amount/quality of maternal care received by handled versus maternally separated pups (48). This suggests that the amount and quality of maternal care, at least in part, mediates effects of handling and maternal separation on functioning of the HPA axis in the offspring.

However, certain findings challenged this theory. For instance, Macri and co-workers (32) reported inconsistencies in the maternal mediation hypothesis. They showed an overall increase in maternal care following both handling and maternal separation. Their findings revealed that directly following maternal separation dams increase their care to such an extent that they fully compensate for the separation time and reach a level comparable to dams of handled pups. Since handled and maternally separated offspring display significantly different endocrine and behavioural stress responses in later-life, it was concluded that maternal care cannot be the only mediator driving the effects of the postnatal manipulations (32, 33).

4. NATURALLY OCCURRING VARIATION IN MATERNAL CARE

4.1 Rodent studies

The most compelling set of evidence for the importance of the amount and quality of maternal care on the development of the stress-regulating system came from studies performed by Meaney and colleagues (49). Employing a non-invasive naturalistic approach, they studied the impact of naturally occurring variation in maternal care on the development of the HPA axis in rodents. This model is based on extreme differences among lactating rats in the frequency of licking and grooming (LG) they provide to their pups. It shows that variation in the amount of maternal LG, a form of tactile stimulation, modulates the development of the structure and function of the neural circuitry underlying stress regulation, emotionality, and cognitive processes (49-54). Reminiscent of the outcome of handling, offspring of high, relative to low LG dams, show decreased behavioural and endocrine responsiveness to stress, reduced emotionality, and enhanced performance in tests of spatial learning (49, 51, 54). These effects are largely reversed with cross-fostering, in which the biological offspring of a high LG mother is cross-fostered to a low LG mother or vice versa. This suggests that variation in maternal care transfers phenotypic differences to the offspring in a non-genetic way (55).

→ *This model is based on the assumption that maternal care is equally distributed over individual pups sharing a litter, such that each individual develops a similar phenotype later in life. In chapter 2 of this thesis this assumption is tested. We hypothesize that the distribution of maternal care directed towards individual pups within a litter is homogenous and therefore results in a uniform 'stress phenotype' in later-life.*

4.2 Human studies

As with animal models, the mediating role of the mother (or another caregiver) in the regulation of the HPA axis of the infant has also been demonstrated in humans (56). Several studies show that when children are exposed to adequate

care, they display diminished cortisol responsiveness, an increased threshold to evoke a cortisol response to various stressors (56), and a better cortisol recovery after stress (i.e. enhanced glucocorticoid negative feedback) (57). This is explained by suggesting that children, under high care-giving conditions, anticipate that a caregiver will protect them and therefore they feel able to cope with a threatening situation (56). Additionally, it has been reported that subtle differences in parenting style are associated with the degree of antisocial behaviour in adolescents (19). Moreover, differences in *within-family* parenting style appear to be associated with variation in antisocial behaviour and depressive symptoms in the offspring (18).

5. EPIGENETIC PROGRAMMING OF THE HPA AXIS

The neuro-endocrine (49), behavioural (51, 58, 59), and cognitive alterations (50, 52) observed in response to naturally-occurring variations in maternal care (60) are suggested to be the consequence of alterations in HPA axis activity, hippocampal glucocorticoid receptor (GR) expression and synaptic plasticity.

A major breakthrough in the field of developmental programming came with the discovery of epigenetic modifications in the promoter area of the GR gene, revealing a mechanism underlying these environmentally driven effects on later-life stress phenotype. It was shown that increased levels of maternal LG during the first week of life alter the methylation pattern of the GR gene in the hippocampus of the offspring (61). These changes persist into adulthood and alter the expression of the GR throughout life via modification of the chromatin structure. Cross fostering of the offspring (from a high to a low LG dam or vice versa) shows a complete reversal of methylation patterns, demonstrating that DNA can be structurally modified (without alterations to sequence) through environmental influences, thus leading to changes in gene expression (61, 62).

The significance of these findings in the field of psychiatry is unclear but recent studies in humans revealed that epigenetic programming of the HPA axis via changes in DNA methylation of GR may occur in human infants born to mothers whom experienced depression during pregnancy (63). Additionally, there are indications of epigenetic regulation of GR in the brains of individuals with a history of adverse childhood experiences whom committed suicide following a stressful life event (64).

6. RESILIENCE

Traditionally, research in the field of developmental programming has focused on the detrimental consequences of stress and far less on the ability to develop resilience to stress or stress-related diseases. Recent findings are challenging this view and suggest that the outcome of early experience is not necessarily deterministic and cannot be perceived as *good or bad*.

6.1 Resilience through matching environments

From an evolutionary perspective, biological mechanisms leading to 'programming' effects are generally meant to be adaptive and not necessarily a substrate for diseases. This is the basis of the 'predictive adaptation plasticity hypothesis' (65-69). This theory is based on the concept that a developing organism responds to cues (e.g. maternal care) in its environment by changing certain aspects of its homeostatic regulation (e.g. HPA axis) in order to produce a phenotype that is highly adapted to its current and anticipated future environment. This concept led to the idea that a high degree of 'mismatch' between the early- and later-life environments accounts for an increased risk to develop diseases in adulthood (66-69). There is much evidence to support this view in the field of metabolic and cardiovascular disorders (67, 70). However in psychiatric research, the validity of this concept is uncertain.

Recent evidence from animal studies however suggests that the concept of 'mismatch' can also apply to the development of individual differences in stress sensitivity. It was recently shown that the outcome of early experiences on stress-related parameters is dependent on later-life environmental context (50, 52). Specifically, it was reported that adult offspring of low LG mothers (considered as a form of adversity) show indeed the expected poor cognitive performance in a low-stress context. However, in a high-stress context their performance was better compared to animals that had received high levels of maternal LG, which in turn were impaired under the same stressful conditions (50, 52). Additionally early deprivation of maternal care (a severe form of adversity) has been reported to result in impaired cognition under low stress but enhanced performance under high stress conditions (71). These findings suggest that the influence of environmental experiences during development might serve as a basis for resilience to stressful challenges in later life.

6.2 Intervention

Interventions, when made at a specific time during development, can mediate the developmental programming of a certain phenotype, as is shown by studies on infants raised in orphanages. These infants have been reported to show changes in cognitive performance (72) and neuronal function in the hippocampus, when compared to never-institutionalized children (73). However, these deleterious effects appear to be reversible when intervention occurs within a certain time window. Placement of institutionalized infants in foster families significantly improves long-term learning and memory performance, with earlier intervention leading to better outcome (74).

The impact of interventions has also been described in animal studies. It has been reported that the cognitive impairment in animals either receiving low levels of maternal LG (75) or being exposed to prolonged periods of maternal separation (76) in early-life can be reversed by exposing them in the peri-pubertal period to environmental enrichment, an effect that might be mediated via structural changes in the hippocampus (77). These findings indicate that even 'adversely' programmed individuals can be 'rescued' by environmental interventions.

→ *In chapters 3 and 5 two intervention strategies to overcome the frequently reported adverse effects of neonatal glucocorticoid exposure will be described*

7. THE STRESS HYPO-RESPONSIVE PERIOD

The outcome of early-life experiences largely depends on the timing, frequency and duration an individual is exposed to particular environmental experiences (78-81). For instance, the timing of handling and 24h maternal separation is crucial for the outcome on adult phenotype, with handling effects being more profound if performed during the early postnatal period as compared to later in the postnatal period (78, 80). This is important since the early postnatal period coincides with onset of the stress hypo-responsive period (SHRP). The SHRP begins several days after birth and terminates around pnd 14 in rodents (28, 45). This period is characterized by very low basal corticosterone (CORT) levels and a reduced ability to show an increase in circulating CORT levels in response to mild stressors that are capable of triggering a profound glucocorticoid response in the adult animal (82). While during the SHRP the neonate's pituitary-adrenal axis is mostly hypo-responsive (83), the central component of the HPA axis does respond to stressors as is revealed by activation of hypothalamic paraventricular (PVN) neurons (84). This hypo-responsiveness of the adrenals is time and stressor specific because more severe stressors have been shown to induce a substantial CORT response (85, 86).

Interestingly, the presence of the mother is highly important in maintaining the SHRP (87, 88). Maternal presence in rodents - resulting in active maternal care and feeding - is suggested to actively regulate the responsiveness of the neonate's HPA axis during the SHRP (82, 89). Adrenocorticotropic hormone (ACTH) and CORT levels slowly increase if pups are separated from the mother, reaching peak levels after 8 h (90-92). The SHRP is disrupted and an adrenal CORT response is more easily activated after exposure to mild stressors and exogenous ACTH administration (91, 93, 94). When certain aspects of maternal behaviour are reinstated during separation, by stroking and feeding of the pups, the effects evoked by separation can be reversed (95)(see figure 1). Therefore maternal presence serves to 'buffer' the impact of stressors on the neonate. There is accumulating evidence that a human analogue to the rodent stress hyporesponsive period exists, emerging in infancy and lasting throughout most of childhood (56).

7.1 Social buffering and attachment learning

The importance of the SHRP and the role of the mother were also illustrated in the context of attachment learning. During the first days of life, rodent pups strongly depend on the mother for survival. They must learn to approach her and exhibit certain behaviours to elicit nursing behaviour from the mother. Since pups do not see or hear during the early postnatal period, attachment to the mother is based on odour learning, supported by a circuitry involving the olfactory bulb

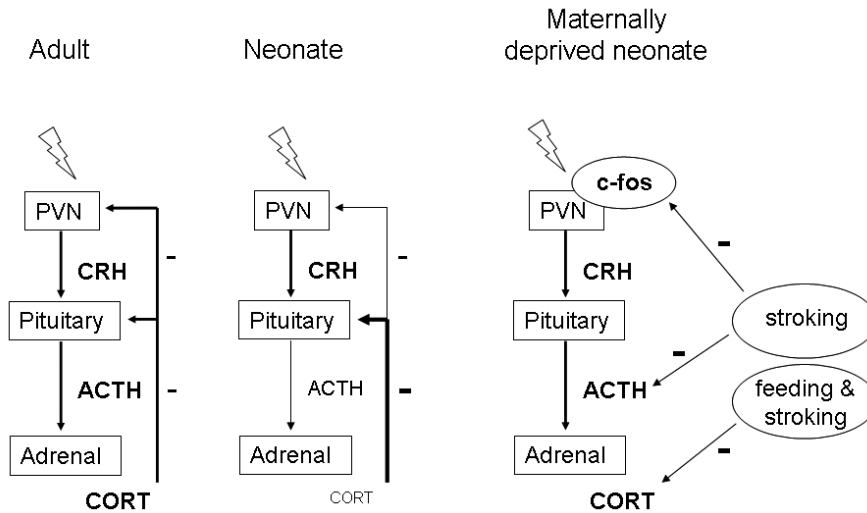

Figure 1. Stress-induced HPA axis activity of an adult, neonate and maternally-deprived neonate rat. During the SHRP the neonate rat shows a central response to stressors, which is not translated to a corticosterone response. The SHRP is characterized by hyporesponsiveness of the adrenals to stress resulting in low and stable levels of circulating corticosterone which is unbound because corticosteroid binding globulin is virtually absent at that age. Also on other levels of the HPA axis there are differences in sensitivity and reactivity compared to an adult animal. Interestingly, the HPA axis of a maternally-deprived neonate is responsive to stressors, showing some resemblance to that of an adult animal and therefore suggesting premature maturation of the stress pathways. However, when certain aspects of the maternal behaviour repertoire are reinstated (by stroking and/or feeding the neonate) during deprivation, several deprivation-induced alterations can be reversed. The size and thickness of symbols and lines represent the magnitude of responsiveness. - depicts suppression of stress-induced activity. PVN: paraventricular nucleus of the hypothalamus; CRH: corticotropin-releasing hormone, ACTH: adrenocorticotropic hormone, CORT: corticosterone.

and locus coeruleus. Pups readily learn to approach the mother based on her smell. Additionally, they learn to approach artificial odours when paired with positive stimuli such as stroking and warmth, and during the very early postnatal period even to negative stimuli such as a shock, indicating that the neonate is programmed for attachment rather than avoidance (96).

Interestingly, this attachment learning only occurs under low CORT conditions. When the neonate reaches the end of the SHRP, and mild stimuli start to elicit a rise in CORT levels, the buffering role of the mother becomes important for maintaining attachment learning. A novel odour paired with a shock will not result in a rise in CORT in presence of the mother, and the pup will learn to approach this odour. However if conditioning takes place in absence of the mother, the pup will display increased CORT levels in response to the shock, which will activate the amygdala, and will result in a shift from odour preference to odour avoidance. Older rodents (weaning age and older), having a mature HPA axis, will always show aversion to odours paired with negative stimuli since they elicit a rise in CORT

levels and subsequently activate the amygdala-fear pathway. Activation of this avoidance learning is obviously an important survival strategy for animals that can no longer depend on their caregiver and have to face the challenges of the world outside the nest (96, 97). However, when this fear pathway is triggered during the very early 'sensitive' postnatal period, due to prematurely elevated CORT levels, there will be long-term consequences for functioning of the amygdala and related pathways with a bias to enhanced activation (Daskakalis et al., submitted).

8. GLUCOCORTICOIDS DURING DEVELOPMENT

The purpose of the SHRP might be to protect the rapidly developing brain from the impact of high levels of glucocorticoids. Appropriate levels of glucocorticoids are necessary for normal development (88, 98, 99). However, not only exposure to high levels of glucocorticoids is disadvantageous, also very low or absent glucocorticoid levels adversely affect development. During normal pregnancy the activity of the maternal HPA axis is dramatically changed, leading to increased circulating glucocorticoid and ACTH levels (100).

The impact of glucocorticoids during development is frequently studied in the context of lung development. Glucocorticoid receptors (GR) are expressed in most foetal tissue and mediate the glucocorticoid action that is essential for survival. GR null mice die several hours after birth because of insufficient lung development and respiratory failure (101). Additionally, animals devoid of the actions of glucocorticoids suffer perinatally from abnormal pulmonary development due to hyper-proliferation and can be rescued by (prenatal) glucocorticoid treatment, a treatment that is obviously ineffective in GR null mice (102, 103).

In prematurely born infants, who frequently display underdeveloped lungs at birth, glucocorticoid treatment can enhance lung maturation (104, 105) by stimulating differentiation of epithelial cells (106). Exogenous glucocorticoid administration during normal development however leads to hypo-proliferation, as well as pulmonary epithelial maturation (107). It appears that glucocorticoid exposure enhances maturation/differentiation at the expense of growth/proliferation, as is reviewed by Bolt (108). These effects can be either beneficial or detrimental depending on the developmental context.

8.1 Synthetic glucocorticoid treatment for prematurity associated respiratory distress syndrome

The initial, and accidental, discovery that antenatal glucocorticoid treatment was associated with accelerated lung maturation (109) led to a first controlled study showing that this treatment prevented respiratory distress syndrome in prematurely born infants (110). Since this important publication numerous reports of randomised controlled trials have been published on this topic (111). Several major health organisations started to recommend the use of antenatal glucocorticoids to reduce the incidence of respiratory distress syndrome (112-114).

Also the postnatal administration of glucocorticoids to attenuate pulmonary inflammation contributing to the pathogenesis of bronchopulmonary dysplasia became common practice (115). By the end of the 1990's glucocorticoid use peaked at around 25% (postnatal) and 60-75 % (antenatal) of all preterm infants (116, 117). However, besides acute beneficial effects leading to reduced mortality and bronchopulmonary dysplasia, there was growing evidence that repeated courses of antenatal (118) and also early postnatal glucocorticoid treatment (119) led to adverse neurodevelopmental effects. Its image of 'magic bullet' changed into 'misguided rocket' (120)(see table 1).

Although long-term follow up studies are scarce (because treated subjects are still relatively young), there are now several reports on the 'long-lasting' outcome of neonatal glucocorticoid treatment showing alterations in cardiovascular, endocrine, immune, motor and cognitive functioning (121-124). Meta-analyses on the lasting effects of this treatment are unfortunately not yet available.

8.2 Impact of neonatal glucocorticoid treatment: rodent studies

To elucidate the neurobiological mechanism underlying the neurodevelopmental side effects reported in human preterm infants, the consequences of neonatal glucocorticoid treatment have been investigated using animal models. The use of rats is especially interesting since rodent pups are born prematurely by nature. The growth spurt of the brain during early postnatal development in rat pups shows similarities with that of human babies during the last trimester of gestation, see Box 1 (137). Since neonatal glucocorticoid treatment is usually administered between 26 and 33 weeks postmenstrual age (last trimester) in the neonatal nursery, the neonate rat pup can be used to study the neurodevelopmental impact of glucocorticoid treatment in the premature infant.

Over the last decade many studies were published on the impact of neonatal dexamethasone treatment in rats. Among the numerous findings were reports on altered social behaviour in adolescence and adulthood (142), impaired spatial learning (144) and hippocampal synaptic plasticity (144, 145), altered endocrine

Table 1. Adverse side effects of glucocorticoid treatment in preterm infants.

	Effect	Reference
Growth	Reduced somatic growth	(125-129)
	Reduced head circumference	(129)
	Reduced gray matter growth	(130)
Motor	Impaired motor performance	(131)
Endocrine	Suppressed HPA activity	(123, 132-134)
Metabolic	Hyperglycaemia	(119, 126, 127, 129, 135, 136)
Immune	Altered Th1-Th2 balance	(123)
Gastrointestinal	Perforation	(119, 126)
Cardiovascular	Hypertension	(119, 126, 129, 135, 136)
Cognition	IQ	(124, 129)

Box 1. Relevance of treatment design for human clinical situation.

Extrapolating findings from animal studies to humans can only be done with great caution. Although there is much variation between species in the complexity of the mature brain and in the timing of neurodevelopmental processes in relation to the timing of birth, there are great similarities between rodents and humans in the sequence of events during brain development. The human brain shows a growth spurt during the last trimester of pregnancy that peaks around birth. In rodents, this growth spurt takes place during the first 10 postnatal days. This suggests that the developmental stage of a rodent brain on postnatal day 1 corresponds to a human brain at the start of the third trimester of pregnancy (137), i.e. a premature infants' brain. Therefore, exposing rodents to glucocorticoids during the first postnatal days can be used as a model to study the neurodevelopmental impact of glucocorticoid treatment in the preterm infant.

Clinical protocols show much variation in timing of treatment initiation (due to timing of preterm birth), duration (1-42 days), as well as cumulative dose (0.2-14 mg/kg) of postnatal dexamethasone treatment. Dosage starts however frequently at 0.5 mg/kg (138, 139). With the current design, a 3-day course of dexamethasone treatment (0.5, 0.3, 0.1 mg/kg), we aimed to deliver a relevant treatment in terms of dosage and timing/duration. Although the cumulative dose seems relatively low, the finding that the rodent is relatively corticosteroid-sensitive compared to man (140), might explain why such severe developmental alterations have been reported in rats using a similar dosage regimen (141-144).

responsiveness to stress (141, 146, 147) and a significant shortening of the lifespan (143, 148, 149). This reduction in lifespan has been associated with heart and kidney failure (143, 149-152). Additionally, immune function has been reported to be affected by neonatal glucocorticoid exposure (153, 154). For an overview of findings in rodents, see table 2.

→ *In chapters 3, 4 and 5 of this thesis the acute and long-lasting impact of neonatal dexamethasone treatment in rats will be described. Additionally, two intervention strategies to overcome the frequently reported adverse effects will be introduced.*

9. SCOPE OF THE THESIS

It is well documented that early-life experiences have an impact on development and aging. The aim of the research described in this thesis is to explore the short- and long-term consequences of two distinct types of early postnatal experiences:

Table 2. Effects of neonatal glucocorticoid treatment in rodents.

	Effect	Drug	Time (days)	Reference
Development				
Body weight	↓	D	1-3 / 1-5 / 3-6 / 4	(142, 145, 146, 155, 156)
Brain weight	↓	D	4 / 3-6 / 7	(147, 155-157)
Cell proliferation	↓	HC	1-4 / 1-7	(158-160)
Adrenal Weight	↓	D	1-5	(146)
Eye opening	↑	D	3-6	(147, 156)
Social behaviour				
Social play	↓	C,D	1-4	(161)
	↑	D	1-3	(142)
Submission	↑	C	3-5	(162)
	↓	D	1-3	(142)
Sexual performance	=	C	1-2	(161)
	↑	D	1-3	(142)
	↓	C	1-3	(163)
Learning and Memory				
Water maze	↓	D	4 / 7 /1-3	(144, 155, 157, 164)
Hippocampal synaptic plasticity	↓	D	1-3	(144, 145, 165)
Passive avoidance	=	D	1-3	(145, 156)
Anxiety				
Elevated plus maze	=	D	1-3	(142)
	Closed arms ↑	D	3-6	(156)
Adult HPA axis				
Basal CORT	=	D	1-3 /1-5	(141, 146)
Stress-induced CORT	Novelty:↓	D	1-3	(141)
	Conditioned fear: =	D	1-3	(141)
	LPS challenge: ↓	D	1-3	(153)
	Crowding: ↑	D	3-6	(156)
	Restraint: ↓	D	1-5	(146)
ACTH-induced CORT	=	D	1-3	(141)
Lifespan				
Survival	↓	D	1-3	(143)

C: corticosterone, HC: hydrocortisone, D: dexamethasone

1) very subtle variations in maternal environment, and 2) exposure to synthetic glucocorticoids. Since the outcome of neonatal glucocorticoid exposure has been reported to be detrimental, we additionally investigated the possibility to reverse these frequently reported adverse effects of glucocorticoid exposure by both pharmacological and behavioural intervention.

Within-litter differences in maternal care
To investigate the consequences of experiencing subtle differences in maternal environment we used an adjusted model of naturally occurring variations in maternal care allowing the study of individual within-litter differences in maternal licking and grooming in Wistar rats. Endocrine responsiveness to an acute novelty stressor was investigated in adolescence and adulthood.

Hypothesis: *Maternal care is equally distributed across littermates, resulting in the development of a uniform stress phenotype within the litter.*

Neonatal synthetic glucocorticoid exposure
To investigate the impact of neonatal exposure to synthetic glucocorticoids, newborn Long Evans rats were injected with dexamethasone. We investigated the consequences of this treatment in early-life, adulthood, middle age and senescence using behavioural and molecular techniques. Additionally, we tested the rescuing potential of behavioural and pharmacological intervention strategies.

Hypotheses:
1. *Neonatal dexamethasone treatment acutely affects brain development*
2. *Neonatal dexamethasone treatment results in long-lasting alterations in endocrine and behavioural reactivity*
3. *These effects can be prevented by*
   I. blocking central GR activation prior to dexamethasone exposure
   II. handling of the neonate during the first 3 weeks of life

10. OUTLINE OF THE THESIS

Chapter 2 describes an adjustment of the original maternal care model as described in section 4.1 which allows the study of individual within-litter differences in maternal care. We report that besides differences in maternal care *between* litters, differences *within* the litter exist. Furthermore, these subtle differences in early maternal environment have long-lasting effects on the offspring's stress phenotype, although in a gender-dependent manner.

Chapter 3 describes the acute central effects of neonatal dexamethasone treatment. We report that hippocampal cell proliferation is acutely, but transiently reduced. The number of astrocytes is reduced one week post-treatment, an effect that can be fully prevented by central GR antagonist pre-treatment, which is proposed as a potential intervention strategy to prevent certain dexamethasone-induced changes in the developing brain.

Chapter 4 describes the long-term effects of neonatal exposure to dexamethasone using several behavioural paradigms. We report that although neonatal dexamethasone treatment leads to developmental alterations, the frequently reported adverse effects on adult phenotype were not observed. It is

suggested that handling of the infant during the postnatal period mediates - and potentially overrides - the outcome of neonatal dexamethasone exposure.

In **chapter 5** this hypothesis is further investigated with the goal to examine the potential of neonatal handling to reverse adverse effects induced by neonatal dexamethasone treatment. We report that the effects of dexamethasone treatment interact with those of neonatal handling in shaping the adult endocrine and behavioural phenotype.

In **chapter 6** all experimental findings are summarized and the relevance of their interactions in shaping the adult phenotype is discussed.

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CHAPTER 2

Within-litter differences in individual mother-infant interaction predict stress phenotype in later-life

Sanne E.F. Claessens, Nikolaos P. Daskalakis, Rixt van der Veen,
Melly S. Oitzl, E. Ronald de Kloet and Danielle L. Champagne

Division of Medical Pharmacology, LACDR/LUMC, Leiden University, The Netherlands

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Development of individual differences in stress responsiveness:
an overview of factors mediating the outcome of early-life experiences

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ABSTRACT

Previous studies have shown that naturally-occurring variation in maternal care during the first week of life predicts stress responsiveness, cognitive performance and emotionality in adult offspring. These findings are based on the assumption that maternal care is distributed equally across pups sharing a litter, such that each individual pup inevitably develops a similar phenotype later in life. Therefore, the current study tests the hypothesis that the distribution of maternal care directed towards individual pups *within* a litter is homogenous and therefore results in a uniform 'stress phenotype' in later-life among individuals within a given nest.

Maternal care directed towards individual pups was observed for 5 h/day during the first week of life. Each pup was daily labeled and weighted, and examined at adolescence and adulthood for its endocrine responsiveness to a novelty stressor using an open field test. Blood samples were collected for analyses of basal and stress-induced corticosterone (CORT) levels. Our data show that: 1) distribution of maternal care is unequal *within* the litter, 2) males receive higher levels of maternal care than females, and 3) basal and stress-induced CORT levels were negatively correlated with the amount of maternal care received.

These findings reveal the impact of individual pup-oriented maternal care on the development of individual differences in stress responsiveness in later life and can help provide more insight into how very subtle early-life experiences within the family unit promote vulnerability or resilience throughout the lifespan.

INTRODUCTION

Both human epidemiological data and animal studies have consistently shown that adverse early-life events are associated with alterations in stress responsiveness as well as impaired cognitive and emotional development (1-6) which may increase the susceptibility to a wide array of both mental and physiological disorders later in life (7-13). The mechanisms underlying these long-term effects of early-life environmental adversity are largely unknown, but are thought to involve alterations in mother-infant interaction. Therefore, amount and/or quality of maternal care are seen as mediating factors for the effects of early environmental adversity on the development of the offspring.

Previous studies using a rodent model of naturally-occurring variation in maternal care indeed have shown a strong mediating effect of maternal care on the offspring's stress responsiveness, emotional and cognitive development (4, 14, 15). This model is based on individual differences among lactating rats in the frequency of maternal licking and grooming (LG) they provide to their pups and has shown that as adults, offspring raised by mothers that naturally display high levels of LG show decreased behavioural and endocrine responsiveness to acute stress compared with offspring of mothers that naturally show lower levels of LG (14, 15). Cross-fostering studies, in which the biological offspring of a high LG mother is cross-fostered to a low LG mother or vice versa, show that this procedure completely reverses the phenotype of the offspring (16), suggesting that maternal care transfers differences in stress responsiveness to the offspring in a non-genetic way.

In addition to stress responsiveness, development of emotionality and cognitive performance in the adult offspring also depends on the amount and quality of maternal care received during early-life. Offspring of high LG mothers showed substantially reduced behavioural fearfulness in response to novelty compared with offspring of low LG mothers (15). Additionally, relative to offspring of low LG mothers, offspring of high LG mothers showed greater hippocampal dendritic (and spine) complexity, synaptogenesis and synaptic plasticity, which are associated with enhanced long-term potentiation (LTP), spatial learning and memory function (4, 17, 18).

Interestingly, due to methodological considerations, the association between maternal care during early-life and phenotype of the offspring in later-life is based solely on maternal phenotype (i.e. high vs. low LG dam) and not on what is experienced by individual pups within the nest. However, recent studies have suggested that pups from the same litter (assumed to have received the same amount of maternal LG) display substantial variation in behavioural phenotype later in life (19). Additionally, we previously reported that a 24 hour maternal deprivation results in the amplification of individual differences in stress responsiveness, rather than having a generalized outcome (20). What makes some individuals more vulnerable or resilient to the impairing effects of maternal deprivation is largely unknown; however we propose that this phenomenon might be mediated by differences in individual mother-infant interactions during early life.

This study, using the Wistar rat as a model, provides a detailed description of the amount and quality of maternal care received by each individual pup *within* the litter during the first week of life. Secondly, we investigated whether variation in maternal care *within* the litter correlates to phenotypic differences in stress responsiveness in the offspring later in life, thus complementing previous studies on variation in maternal care *across* litters.

METHODS

Animals

20 female Wistar rats (6 weeks, 200 g) and 10 male Wistar rats (6 weeks, 250 g) obtained from Harlan (Horst, the Netherlands) were used as breeders. After a habituation period of one week, two females were mated with one male in type 4 polycarbonate cages (59x38x20cm) containing sawdust bedding and tissues. Food (RM3, Special Diet Services, Witham, Essex, UK) and water (8 ml 25% HCl /10 L tap water) were provided *ad libitum*. Animals were maintained on a 11-h light : 13-h dark cycle with light on at 08.30h, in a temperature (21+/- 2°C) and humidity (55 +/- 5%) controlled room. After breeding, pregnant females were individually housed in Plexiglass cages (55x30x44cm) that were customized to allow a clear view of all activity within the cage, despite the presence of sawdust bedding and tissues. Females were checked daily for presence of pups. If pups were present, the day of birth for that particular litter was defined as postnatal day 0 (pnd 0). On pnd 1, litters were culled to 8 pups (4 males and 4 females). Cages were not cleaned until pnd 10 and after that once weekly. After weaning (pnd 21), offspring was housed in same-sex, same-litter groups in type 4 polycarbonate cages (59x38x20cm) until time of testing.

Animal experiments were approved by the Local Committee for Animal Health, Ethics and Research of Leiden University and carried out in accordance with European Communities Council Directive (86/609/EEC).

Marking

In order to discriminate between individual pups within the litter, all pups were daily marked using a non toxic, odourless surgical marker (Codman, via Johnson & Johnson, Amersfoort, the Netherlands). The marking of the pups always took place between 08.30h and 10.30h in the morning. The procedure took about 7 min per litter and consisted of removing all the pups from the mother and marking them gently one by one after recording their body weight. A schematic of the marking system is depicted in Fig 1. Upon completion of the marking procedure, all pups were immediately returned to their mother.

Maternal Behaviour

The maternal behaviour of each dam was observed and scored for five 60 minutes periods per day during the first 7 days post partum using a procedure as described by Champagne and colleagues (21). Observations were performed

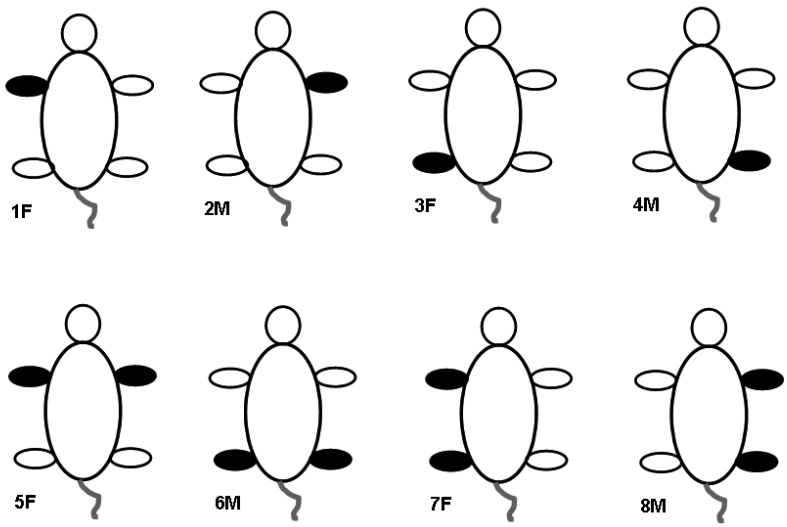

Fig 1. Marking system used to discriminate between individual littermates. Black areas indicate body parts that were marked. M = male, F = female.

at three periods during the light phase (10:00, 13:00, 17:00) and two periods during the dark phase (07:00 and 20:00) of the cycle. Within each 60 min observation period the behaviour of each mother was scored every 3 minutes (20 observations per period, 100 observations per day, 700 observations for the first 7 days postpartum, see Fig 2). The following maternal behaviours were scored: a) frequency of licking and grooming (LG) of the pups (whole body LG and anogenital LG), b) frequency of arched back nursing/blanket nursing/passive nursing, c) mother away from nest/no maternal contact. The data were analyzed as the percentage of observations in which animals displayed one of the behaviours described above. Furthermore, for every maternal behaviour scored, it was noted to which pup(s) it was directed. The data for these individual observations were analyzed as the percentage of observations in which each pup received one of the maternal behaviours described above. The total percentage of LG received by individual pups was used to categorize them. For each litter a LG 'family mean score' was calculated and pups that displayed a LG score of at least 1 standard deviation above or below the family mean were selected as high and low LG pups, respectively.

Endocrine responsiveness to novelty stress

Selected high and low LG animals (n = 13) were tested for their responsiveness to acute novelty stress in adolescence (pnd 28) and adulthood (4,5 months). All animals were individually housed two days prior to testing (to reduce the effect of testing-order within the cage) and returned to their home cage directly after testing. The novelty stressor consisted of 10 min exposure to a black circular open

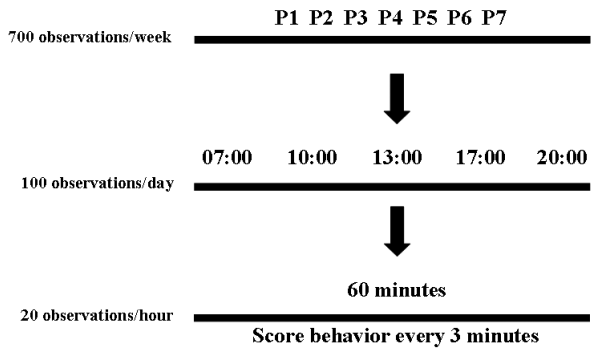

Fig 2. Observation schedule. Adapted from: Champagne *et al*, 2008. P1: postnatal day 1, P2 : postnatal day 2, etc.

field apparatus (diameter: 90 cm, height: 40 cm) in a dimly lit room (approximately 30 lux floor level). Two animals were tested at the same time and in the same testing room using two separate open field setups. All testing took place between 09.00 and 13.00h, and each test apparatus was cleaned thoroughly between animals using 70% ethanol.

Blood sampling for endocrine measurement

Blood samples were obtained from the tail vein, by a small incision with a razorblade at three different time points: a) basal, one day before novelty exposure, b) 10 min after onset of novelty stress, i.e. directly after termination of stressor, and c) 30 min after onset of novelty stress, i.e. 20 min after termination of stressor. Blood was collected in small EDTA coated tubes (Microvette CB 300 K2E, Sarstedt, Numbrecht, Germany). Plasma was obtained by 15 min centrifugation at 13000 rpm at 4°C and subsequently stored at -20°C until further analysis.

Plasma corticosterone determination

Plasma corticosterone (CORT) concentrations were measured in-duplo using radioimmunoassay (RIA) kits containing ^{125}Iodine labelled CORT (MP Biomedicals, Asse-Regelem, Belgium). All samples were analysed in one session to exclude inter-assay variability.

Data analysis

Statistics were performed using SPSS for Windows (version 14.0; SPSS, Chicago, IL). One-way ANOVA was performed to determine significant differences between high and low LG animals, and between males and females. Graphs were plotted using Prism Graph Pad software.

RESULTS

Maternal licking and grooming

Distribution of maternal LG received by individual pups in each of the 7 litters (with appropriate litter composition) studied is depicted in figure 3 and shows

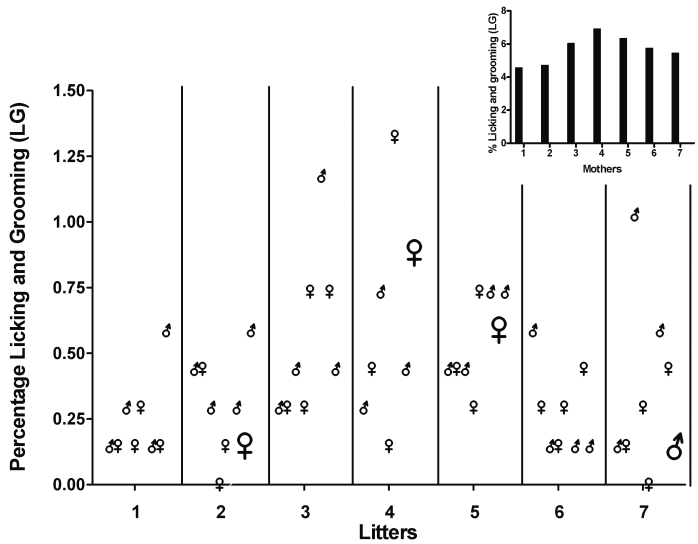

Fig 3. Distribution of maternal licking and grooming (LG) received by individual pups for each litter (1-7). Shown is percentage of observations (pnd 1-7) during which each individual pup received maternal LG. Male pups are indicated with ♂, female pups are indicated with ♀. Selected high (n = 9) and low LG (n = 4) offspring within the litter (that displayed a LG percentage of at least 1 standard deviation above or below the family mean, respectively) are indicated with enlarged symbols**.**
Inset: Total percentage of LG displayed by each mother.

that maternal LG is unequally distributed among pups *within* the litter and that particular pups clearly received higher levels of LG compared to their littermates. However there is variation in the amount of inequality between different litters. Mothers from litters 1, 2, 5, and 6 appear to distribute their care more equally compared to mothers from litters 3, 4, and 7, which show a more heterogeneous distribution of LG.

Gender preference

Additionally, we report that mothers show a preference for male over female offspring ($F = 4.31$; $p < 0.05$; $n = 55$; Fig 4). In the current study, 6 out of 7 mothers spend significantly more time providing LG to male compared to female offspring.

Endocrine responsiveness to novelty stress

Endocrine response to novelty stress, both at adolescence and adulthood was investigated in animals receiving either high or low levels of maternal LG during the first week of life (see Fig 5).

Both basal and stress-induced CORT levels in adolescent animals are associated with individual differences in maternal LG received during early-life. Thus, animals that received high levels of maternal LG *within* litter show significantly lower basal

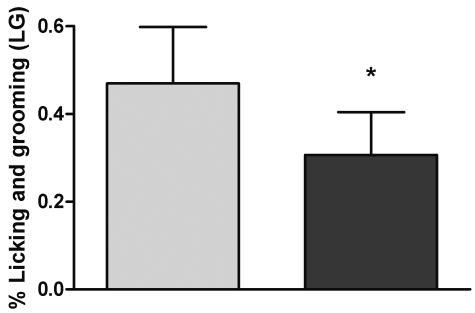

Fig 4. Percentage (mean ± SEM) of maternal licking and grooming (LG) received by male (light grey) and female (dark grey) offspring. In 6 out of 7 litters (86%), males (n = 31) received higher levels of maternal LG compared to females (n = 24). * = $p < 0.05$.

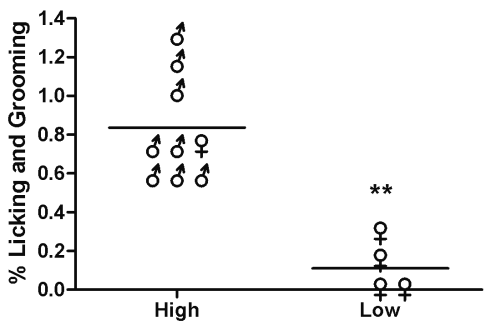

Fig 5. Percentage of licking and grooming (LG) received during the first 7 days postpartum for selected high and low LG pups taken from distinct litters. Male pups are indicated with ♂, female pups are indicated with ♀. The selected high and low offspring significantly differ in the mean percentage of LG ($p < 0.001$). Horizontal lines indicate the group mean. Mean ± SEM high LG group (n = 9: 8 males, 1 female) = 0.83 ± 0.09 and mean ± SEM low LG group (n = 4: all female) = 0.11 ± 0.07.

($F = 5.09$; $p < 0.05$) as well as stress-induced (10 min and 30 min after onset; $F = 4.93$; $p < 0.05$; $F = 5.56$; $p < 0.05$ respectively) CORT levels when compared to animals that received low levels of maternal LG (Fig 6a).

Similar to what is observed in adolescence; the amount of maternal LG received in infancy also appears to predict CORT levels in adulthood. Animals that received high levels of maternal LG *within* litter show significantly lower stress-induced (10 min and 30 min after onset; $F = 61,99$; $p < 0.001$; $F = 14,05$; $p < 0.01$ respectively) CORT levels when compared to animals that received low levels of maternal LG (Fig 6b). Due to technical issues, we are unable to show basal CORT levels of the animals in adulthood.

DISCUSSION

The present study provides a detailed description of the naturally-occurring variation in maternal care received by individual pups *within* a litter over the first week of life. The hypothesis was tested that maternal care is equally distributed among individual pups within the litter and therefore results in a uniform stress phenotype across littermates. We report that: 1) maternal care was unequally distributed among individual littermates, leading to the occurrence of high and

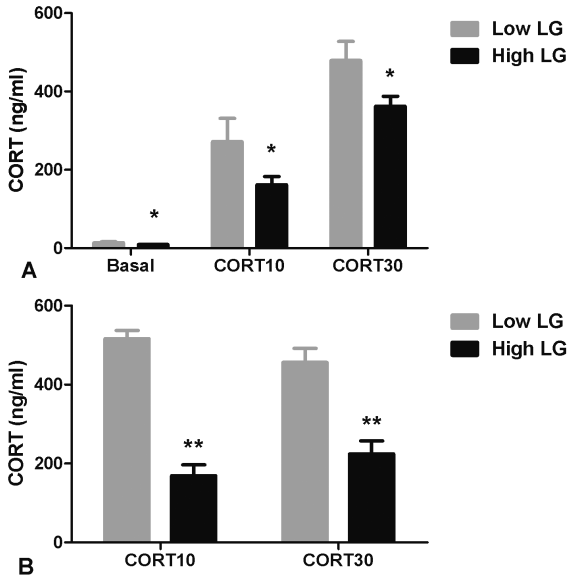

Fig 6. Plasma corticosterone (CORT) levels of rats characterised as high or low LG within the litter, expressed as mean ± SEM. * $p < 0.05$; ** $p < 0.01$. Selected high (n = 9) and low LG (n = 4) animals were tested for their endocrine responsiveness to acute novelty stress in (A) adolescence (age pnd 28) and (B) adulthood (age 4.5 months). CORT levels were significantly more elevated in low LG compared to high LG offspring at 10 min (CORT10) and 30 min (CORT30) after onset of acute stress. Due to technical issues, we are unable to show basal CORT levels of the animals at adulthood; therefore in figure 6B only stress-induced CORT levels are displayed.

low LG offspring within a given litter; 2) LG was biased towards male offspring, leading to the fact that most high LG pups were of the male gender; 3) within-litter differences in maternal LG were negatively correlated with basal and stress-induced plasma CORT levels in adolescence and adulthood, although in a gender-dependent manner.

Individual differences in mother-infant interaction

The present study demonstrates that maternal care is not equally distributed across individual pups sharing a litter, meaning that the mother consistently spends more time caring for certain pups compared to others. However, in previous maternal care studies the behaviour of the mother was observed regardless of which pup the behaviour was directed at (4, 14, 15). In studies using this 'whole litter' paradigm it has been assumed that all pups sharing a litter receive the same amount of care, leading to the hypothesis that all individual littermates inevitably develop a similar phenotype in adulthood, although substantial variation in adult behavioural phenotype has been reported between littermates (19). A more recent study showed that individual differences in maternal LG were correlated with hippocampal synaptic plasticity and glucocorticoid receptor mRNA expression (22). These findings suggest that very subtle differences in *non-shared* maternal LG (up to 10 fold smaller than previously reported differences in LG used to characterize maternal behaviour directed towards the whole litter (21)) appear to have comparable predictive value for later-life phenotypic outcome. Although further studies are warranted to understand these observations, the findings suggest that results from whole litter observations should be carefully interpreted.

Underestimation of individual differences in LG

We showed that the amount of maternal LG is associated with basal and stress-induced CORT levels during adolescence and adulthood. This is reminiscent of previous studies using whole litter observations that have also shown the impact of naturally-occurring variation in maternal LG on endocrine response to acute stress (14, 23). The present study shows that even more subtle naturally-occurring *within-litter* differences in mother-infant interaction are associated with variation in stress responsiveness. However, the differences we observed using this paradigm might be too subtle to reveal significant interactions between LG received in infancy and behavioural responses to novelty stress in adulthood (data not shown); an effect that has been reported using the whole litter paradigm (15, 16).

Wistar dams display overall relatively low levels of maternal care compared to Long Evans dams that are frequently used for studies on the effects of maternal LG (21). Therefore the average levels of LG (i.e. 6%) displayed by Wistar dams in our study would be equivalent to levels displayed by low LG Long Evans dams. In our study, we observed that Wistar dams on average delivered 40 LG events over the 7 day observation period (700 observations). From the pup's perspective, this might represent on average 5 LG events per individual pup. Such scores might appear too low to reliably categorize individual littermates as low or high LG offspring. We acknowledge that this represents a limitation of our experimental approach, which leads to an underestimation of the occurrence of pup-directed LG events. In the future, this issue can be resolved by increasing the amount of observations per day to achieve a more reliable representation of the actual differences in *non-shared* maternal LG.

Gender preference

It is noteworthy that for most of the litters studied, dams displayed a strong gender preference for male over female offspring. Such a preference of the mother for male pups has been shown before (24, 25) and is proposed to be regulated by testosterone, pheromones, and urinary odour (26, 27). As a consequence, this gender preference resulted in a bias in the distribution of males and females over the selected low (exclusively females) and high LG (almost exclusively males) groups. Since glucocorticoid levels and corticosteroid binding globulin levels in the blood are modulated by fluctuations in sex hormone levels (28) the association between early-life maternal LG and later-life HPA axis activity has to be carefully interpreted. However, we report an association between neonatal LG and glucocorticoid levels as early as pnd 28, when the regulatory role of sex hormones on HPA axis functioning is assumed to be low compared to that in adulthood (29). Gender differences in endocrine responsiveness on pnd 28 have been shown in some (30, 31) but not all (32) studies. Because of the small sample size, we were unable to reveal a within-gender effect of maternal LG on glucocorticoid levels. Future studies using a higher number of animals and including ovariectomized female offspring could reveal the exact role of gender-dependent within-litter differences in maternal LG during early-life on the development of stress phenotype in later-life.

Introduction of early handling

In order to score individual LG, a daily marking procedure was used to label and identify individual littermates. Such a procedure inevitably introduces a substantial amount of handling. This implies that besides studying the impact of subtle differences in maternal care within the litter, we have to take into account the well-known effects of handling (33-36). The daily handling/marking procedure potentially altered maternal care directed towards the offspring. However we believe that this bias is distributed to a similar extent to all litters. Furthermore, since we observed a substantial amount of variation between littermates in terms of stress responsiveness, we suggest that *non-shared* experiences in LG rather than the *shared* experience of handling/marking mediate the effects observed here. This scenario is not unprecedented and has been previously proposed by Macri and co-workers for variation in *shared* maternal care (37, 38).

Early-life experiences within the family unit promote development of individual differences

It is well-known that the quality of early family environment can serve as a major source of vulnerability in later life. After both human and animal studies have reported that early-life adversity is associated with alterations in stress responsiveness (39, 40) which may increase the risk for developing various disorders later in life (41, 42), it has now been assumed that this effect is at least partly mediated by parental influence on the development of the stress-regulating system (43-45). Subtle differences in parenting style could lead to dysregulation of HPA responsiveness, which subsequently could promote the development of illness (46-48). Human studies have reported that parenting style not only varies between families but also within the family. These *non-shared* factors, such as variation in individual parent-child interaction might have higher predictive power for the development of stress-related pathologies in later-life compared to the *shared* family environment (49, 50).

The present study is to our knowledge the first to report the impact of within-family differences in parenting in rodents on the development of individual differences in stress responsiveness in later life and can help to provide more insight into how very subtle early-life experiences within the family unit promote vulnerability or resilience throughout the lifespan.

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CHAPTER 3

Acute central effects of neonatal dexamethasone treatment: towards a rescue strategy

Sanne E.F. Claessens[1], Joseph K. Belanoff[2], Paul J. Lucassen[3], Danielle L. Champagne[4,5], E. Ronald de Kloet[1]

[1]Department of Medical Pharmacology, LACDR/LUMC, Leiden University, The Netherlands, [2]Corcept Therapeutics, Menlo Park, USA, [3]SILS-CNS, University of Amsterdam, The Netherlands, [4]Institute of Biology, Department of Integrative Zoology, Leiden University, The Netherlands, [5]Institute for Water and Wetland Research, Department of Organismal Animal Physiology, Nijmegen University, The Netherlands

Submitted

ABSTRACT

Dexamethasone (DEX), a synthetic glucocorticoid, is widely used to wean preterm infants from the ventilator. Despite the important short-term benefit on lung function, there is growing concern about the long-term outcome of this treatment, since follow-up studies of preterm infants have shown pervasive adverse neurodevelopmental effects.

Since the mechanism underlying these neurodevelopmental impairments is largely unknown, the aim of the present study was (i) to investigate the acute effects of neonatal DEX treatment on the developing brain; and (ii) to block the effects of DEX in the brain by central administration of the glucocorticoid receptor (GR) antagonist mifepristone.

Long Evans rat pups were injected subcutaneously with tapering doses of DEX or saline (SAL) on postnatal days (pnd) 1, 2 and 3. Separate groups received intracerebroventricular injections with mifepristone prior to DEX treatment. On pnd 4 and 10 pups were sacrificed and brains collected for analysis of cell proliferation (Ki67) and gliosis (GFAP).

We report that neonatal DEX treatment reduced hippocampal cell proliferation on pnd 4 and caused a significant reduction in the number and density of astrocytes within various brain areas on pnd 10. These effects could be partially prevented by GR antagonist pre-treatment. These findings represent a *proof of principle* for the use of central GR antagonist pre-treatment as a potential intervention strategy to block postnatal DEX-induced alterations in brain development and function.

INTRODUCTION

Preterm birth is the leading cause of perinatal morbidity and mortality in developed countries (1). Although the prevalence of preterm birth has increased over time, survival rates are going up significantly because of technological advances (2). However, preterm infants are still at increased risk for neurodevelopmental impairments and other complications compared to their term counterparts. With most organs being immature in the preterm, lung development especially suffers from prematurity and is frequently associated with morbidity such as respiratory distress syndrome (RDS) and bronchopulmonary dysplasia (BPD). Glucocorticoids (GC) are the drug of choice to accelerate lung maturation and wean infants from the ventilator. Several studies have indeed shown beneficial effects of GC treatment (3) on acceleration of lung maturation and a decreased incidence and severity of BPD. However others fail to do so or show only modest effects (4).

Moreover, follow-up studies of prematurely born infants treated with GC have shown pervasive adverse neurodevelopmental effects (5, 6). Randomized placebo-controlled trials have shown that GC treatment leads to an increased incidence of cerebral palsy resulting in a higher level of neurodevelopmental impairment (5), poor motor skills and lower IQ scores compared to control preterm infants (7). Imaging studies have further revealed a significant reduction in total brain and cerebellar volume at term age in GC-treated premature infants when compared to non-treated preterm infants and control term infants (8, 9).

These alterations in cerebral volume might structurally underlie the cognitive and motor impairments reported in GC-treated infants. Therefore, there have been growing concerns as to whether the short-term benefits of GC treatment outweigh the adverse side effects leading to neurodevelopmental impairment (10). Supporting the evidence from human studies, data from rodent studies have now demonstrated that neonatal GC treatment results in long-lasting alterations in cognitive performance and hippocampal function (11-14), social behaviour (15), stress responsiveness (16, 17) and eventually may even lead to a significant shortening of the lifespan (14, 18).

Here, we aim to investigate the *acute* impact of neonatal GC treatment on the developing brain in rats. We describe its effects on markers for glial activation and cell proliferation at two different time points after administration, 24 hours and 7 days post-treatment respectively. Additionally we propose a potential intervention strategy to block the acute effects of GC treatment on the developing brain.

METHODS

Animals

Adult female and male Long Evans rats from our breeding population were used as breeders. Two females were mated with one male for 10 days in type 4 polycarbonate cages (59x38x20cm) containing sawdust bedding and tissues. Food (RM3, Special Diet Services, Witham, Essex, UK) and water (8 ml 25% HCl /10 L tap

water) were provided *ad libitum*. Animals were maintained on a 11-h light : 13-h dark cycle with lights on at 08.30h, in a temperature (21± 2°C) and humidity (55 ± 5%) controlled room. After breeding, pregnant females were housed individually. Females were checked daily for presence of pups. If pups were present, the day of birth for that particular litter was defined as postnatal day 0 (pnd 0). On pnd 1, litters consisting of 8-12 pups with an appropriate gender distribution (40-60 % male) were selected. If a litter did not meet these criteria, the litter was excluded from the study; no culling was performed. Cages were not cleaned until time of sacrifice. Animal experiments were approved by the Local Committee for Animal Health, Ethics and Research of Leiden University and carried out in accordance with European Communities Council Directive (86/609/EEC).

Drug treatment

Pups were randomly assigned to one of six treatment groups (see table 1) according to a within-litter design. Males and females were equally distributed over the treatment groups. Pups in the DEX groups were subcutaneously (SC) injected with dexamethasone-21-phosphate (Sigma Aldrich, Zwijndrecht, The Netherlands) on pnd 1 (0,5 µg/g body weight), pnd 2 (0,3 µg/g) and pnd 3 (0,1 µg/g). Pups in the SAL groups were injected with equivalent volumes of sterile and pyrogen free saline (SAL). Groups 3-6 received bilateral intracerebroventricular (ICV) injections with either vehicle (VEH: 0.4% Tween 80 (Sigma) in SAL) or the GR antagonist mifepristone (50 ng/µl VEH, Corcept Therapeutics, Menlo Park, USA) 30 minutes prior to DEX/SAL administration. Since mifepristone was administered bilaterally, the total dose per day was 100 ng dissolved in 2 µl VEH (1 µl per hemisphere). ICV injections were performed according to (19) reaching a success rate of > 90%. Custom made syringes and needles were used for both SC and ICV injections (Hamilton, Germany). All pups were daily marked using a non-toxic, odourless marker in order to distinguish the littermates assigned to different treatment groups. Daily marking was essential since marks did not last long due to maternal licking and grooming of the pups.

Table 1. Description of experimental groups.

Group	SC	ICV	N
1	SAL	-	11
2	DEX	-	10
3	SAL	VEH	11
4	DEX	VEH	11
5	SAL	MIF	12
6	DEX	MIF	13

SC: subcutaneous, ICV: intracerebroventricular, SAL: saline, DEX: dexamethasone, VEH: vehicle, MIF: mifepristone.

Procedure

Pnd 1-3: The daily procedure consisted of removing all pups from the nest followed by transfer to an adjacent room, where the cage was placed on a heating pad. If applicable, first the VEH/mifepristone ICV injection was given. Then body weights (BW) were recorded, followed by marking of the pups and administration of the SC injection. After the procedure (which took ~ 35 minutes per litter) all pups were immediately returned to the home cage.

Pnd 4-10: The daily procedure consisted of removing all pups from the nest followed by transfer to an adjacent room. BWs were recorded, followed by marking of the pups. After the procedure (which took ~ 8 minutes per litter) all pups were immediately returned to the home cage. Besides these manipulations (between 9.00 and 13.00h) litters were left undisturbed.

Tissue preparation

To determine effects of neonatal DEX treatment on glial activity and cell proliferation, pups were sacrificed on pnd 4 or 10 (24h and 7 days post-treatment respectively). On the day of sacrifice animals were anesthetized by an intraperitoneal injection with pentobarbital sodium salt (Nembutal, A.U.V., Cuijk, The Netherlands; 1 ml/kg BW) and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde (PFA) in phosphate buffer (PB, 0.1M, pH 7.4). Brains were post-fixed overnight in the skull at 4°C. The next day they were carefully removed from the skull, washed and cryoprotected by equilibration in a buffered 30% sucrose solution. Afterwards brains were snap frozen in ice-cold isopentane and stored at -80°C until further processing.

Brains were sectioned coronally (30 µm) using a cryostat and collected and stored in an antifreeze solution (30% ethylene glycol, 20% glycerol, 0.02 M Na_2HPO_4, 6.6 mM NaH_2PO_4) at -20°C until further processing.

Immunohistochemistry

Free-floating immunohistochemistry for Glial Fibrillary Acidic Protein (GFAP, polyclonal rabbit anti-GFAP, DAKO, 1:1000) was used to determine glial activity in the corpus callosum (CC) and dentate gyrus (DG). The primary antibody was amplified by biotinylated goat anti-rabbit (Santa Cruz, 1:1500), and avidin-biotin enzyme complex (ABC-Kit, Elite Vectastain, Brunschwig Chemie, 1:200). Chromogen development was done with diaminobenzidine (DAB; 0.05% in 0.1M PBS, 0.06% H_2O_2), after which sections were carefully mounted on Superfrost Plus slides (Menzel-Gläser, Germany).

Immunohistochemistry for Ki-67 (polyclonal rabbit anti-Ki67; Novocastra, 1:2000) was used to determine cell proliferation in the DG of the hippocampus as described before (Oomen et al., 2010). The primary antibody was amplified by biotinylated goat anti-rabbit (Vector Laboratories, 1:200), avidin-biotin enzyme complex (Elite Vectastain ABC kit, Brunschwig Chemie, 1:1000) and tyramide (1:500; 0.01% H_2O_2). Subsequent chromogen development was done with diaminobenzidine (20 mg/100 ml Tris buffer, 0.01% H_2O_2).

Stereological quantification

For quantification of the number of GFAP positive cells and density of GFAP staining in CC and hilus of the DG, pictures from these brain areas were taken using a light microscope (Leica DM6000B). Photoshop (Adobe) was used to convert pictures to grayscale. Grayscale pictures were then analyzed with ImageJ (Version 1.41) for optical density of GFAP (correcting for corresponding background optical density) and for quantification of GFAP positive cells. Pictures showing substantial tissue damage were removed from the analysis. On average, 3 sections per animal were used for CC quantification and 7 for DG quantification. Due to technical limitations it was not possible to discriminate between individual astrocytes in the pnd 4 sections. Therefore, for this time point only optical density measurements are shown. However, in the pnd 10 sections both the number of GFAP positive cells and optical density of GFAP were measured.

For quantification of Ki67 positive cells in the DG of the hippocampus, a stereological quantification procedure was performed in every 6th coronal section along the rostrocaudal axis, i.e. a total of 8 sections per animal. Total numbers of Ki67 positive cells were quantified by systematic random sampling performed with the Stereo Investigator system (MicroBrightField, Germany). As Optical Fractionator settings for sampling of pnd 10 sections a grid size of 125 x 125 µm and a counting frame of 30 x 30 µm were used, which resulted in an average count of 300 markers per animal. For analysis of pnd 4 sections a grid size of 75 x 75 µm and a counting frame of 30 x 30 µm were used, which resulted in an average count of 200 markers per animal. DG surface area and volume was determined according to Cavalieri's principle using the Stereo Investigator system.

Data Analysis

All statistics were performed using SPSS 17.0 for Windows. Data are presented as mean ± SEM. Since the data did not show a significant effect of gender, data from males and females were pooled. Due to the limited capacity of our immunohistochemistry setup, analysis of the samples occurred over multiple experimental runs. To correct for inter-run variation we normalized all output against the mean of either the SAL or the VEH SAL samples in the relevant run. This way, for each experimental group, the difference with its relevant control group (either SAL or VEH SAL) is shown in percentages.

Differences between SAL and DEX were analyzed using T-test. Differences between the ICV treated groups were analyzed using two-way ANOVA with ICV treatment (VEH or mifepristone) and peripheral SC treatment (SAL or DEX) as between-subjects factors.

Body weight data were analyzed using Repeated Measures ANOVA with 'pnd' as within-subjects effect and 'treatment' as between-subjects effect.

RESULTS

Body weight pnd 1-10

Neonatal DEX treatment significantly reduces BW gain during the early postnatal period ($p < 0.001$; fig. 1A). This reduction is not normalized by central mifepristone pre-treatment (fig. 1B).

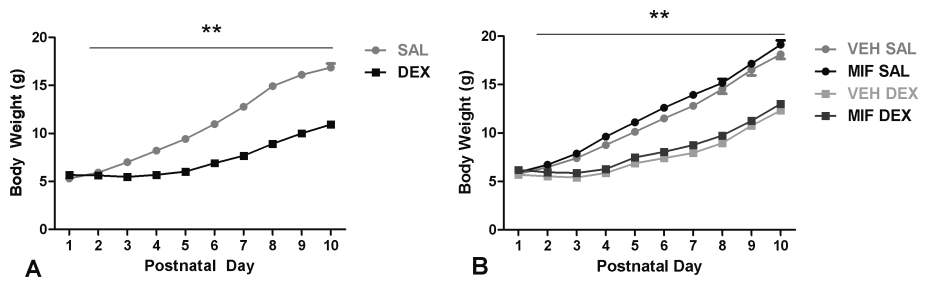

Fig 1. Body weight on pnd 1-10 of SAL and DEX treated animals without (A) or with (B) ICV treatment. DEX treatment significantly reduced body weight; this effect is not prevented by central mifepristone pre-treatment. ** $p < .001$

Glial activity pnd 4

24h post treatment, DEX treatment did not affect optical density of GFAP expression in the CC (fig. 2A). Also in the ICV treated groups (fig. 2B) there are no significant differences between treatment groups. However, there is a tendency towards reduction of GFAP expression in DEX compared to SAL treated groups ($p = 0.072$). Interestingly, mifepristone pre-treatment appears to result in a slight (not statistically significant) increase in GFAP expression compared to VEH treatment ($p = 0.096$). No significant interaction between the SC and ICV treatment was found.

In the hilus of the hippocampus, no main effects of, or interaction between, SC and ICV treatment were observed (fig. 2C & D).

Glial number and activity pnd 10

Although we did not observe an acute effect of DEX treatment (under basal, non-ICV conditions) on GFAP expression, 1 week post treatment DEX resulted in reductions in both density of GFAP expression ($p = 0.023$, data not shown) and number of GFAP positive cells in the CC ($p = 0.003$; fig. 3A). In the hilus, DEX treatment resulted in a trend towards reduced density ($p = 0.068$, data not shown) and significantly reduced the number of GFAP positive cells ($p = 0.002$; fig. 3B).

In the ICV treated groups, we observed a significant interaction between SC and ICV treatment in CC ($p = 0.001$) indicating that the observed reduction in the VEH DEX group is fully normalized by central mifepristone pre-treatment (fig. 3C). There is a trend towards an interaction between SC and ICV treatment in the hilus ($p = 0.080$, fig. 3D). Due to enhanced variation these interactions do not reach statistical significance in the optical density measurements (data not shown).

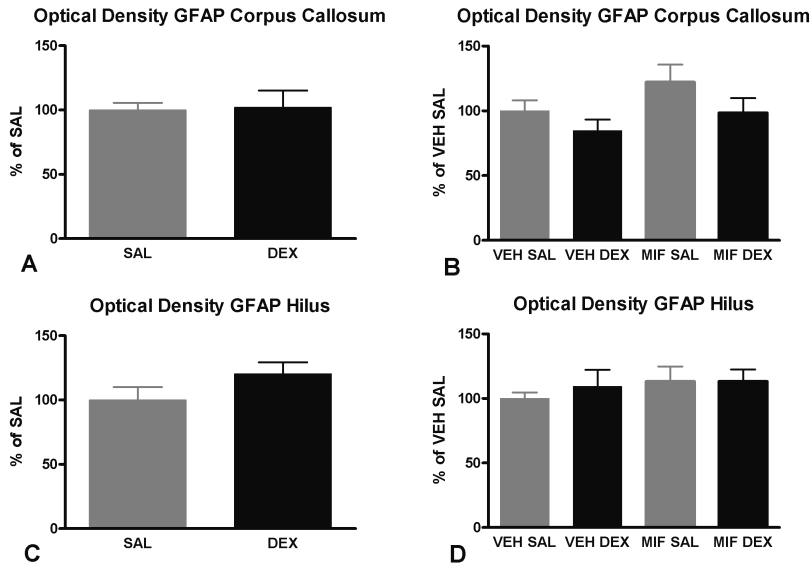

Fig 2. Optical density of Glial Fibrillary Acidic Protein (GFAP) staining in Corpus Callosum (A and B) and hilus of the hippocampus (C and D) 24h post treatment, i.e. on pnd 4.

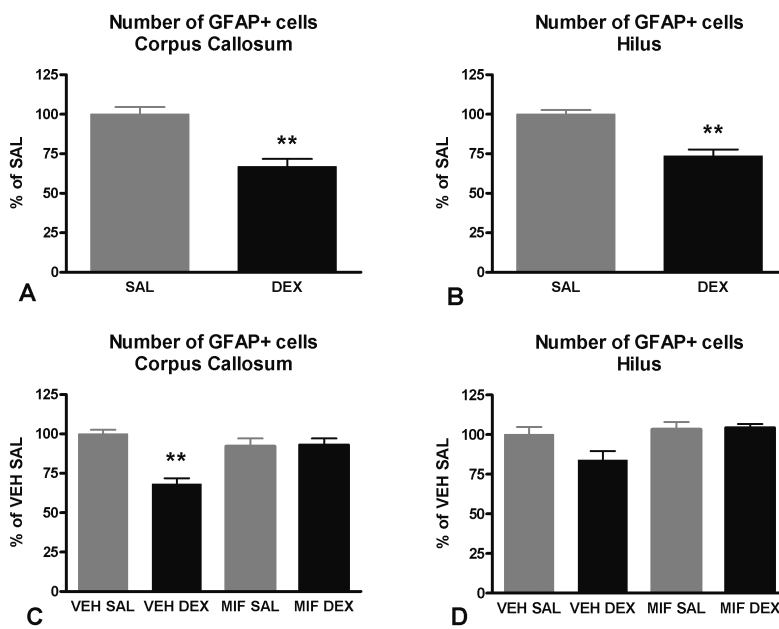

Fig 3. Number of Glial Fibrillary Acidic Protein (GFAP) positive cells in the Corpus Callosum (A and C) and Hilus of the hippocampus (B and D) 7 days post treatment, i.e. on pnd 10 in SAL and DEX treated animals with (C and D) or without (A and B) ICV treatment. ** in A and B: DEX compared to SAL $p < .01$. ** in C: interaction between SC and ICV treatment $p < .01$.

Cell proliferation pnd 4

DEX treatment significantly reduced the total number of proliferating cells in the DG of the hippocampus 24h post treatment ($p = 0.007$, fig. 4A) as well as the volume of the DG ($p = 0.001$, fig. 4B). Additionally, after correction for differences in volume (total number of Ki67+ cells/DG volume) we observed a trend ($p = 0.10$, fig. 4C) towards a reduction in density of proliferating cells in the DG in the DEX group.

In the ICV treated groups, DEX also significantly reduced the total number of proliferating cells in the DG of the hippocampus ($p < 0.001$, fig. 5A) and the volume of the DG ($p = 0.001$, fig. 5B). These reductions were not normalized by central mifepristone pre-treatment. Additionally, the density of proliferating cells was lower in all DEX treated groups ($p = 0.016$, fig. 5C). Although the interaction between ICV and SC treatment does not reach statistical significance there appears to be a tendency towards normalization by central mifepristone pre-treatment (fig. 5C).

Cell proliferation pnd 10

Seven days post treatment the reduction in total number of proliferating cells in the DG (as was observed 24h post treatment) is normalized in the DEX treated group towards control (SAL) levels (fig. 6A). Although the volume of the DG appears smaller in the DEX group (fig. 6B), and the density of proliferating cells higher (fig. 6C), these effects do not reach statistical significance.

Also in the ICV treated groups, group differences that were observed 24h post treatment had normalized to control levels 1 week post treatment (fig. 7A, B & C).

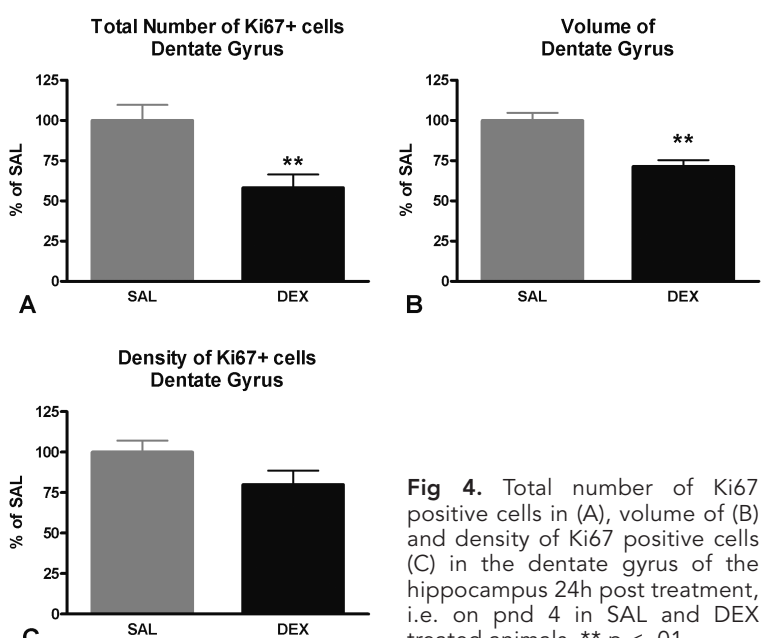

Fig 4. Total number of Ki67 positive cells in (A), volume of (B) and density of Ki67 positive cells (C) in the dentate gyrus of the hippocampus 24h post treatment, i.e. on pnd 4 in SAL and DEX treated animals. ** $p < .01$.

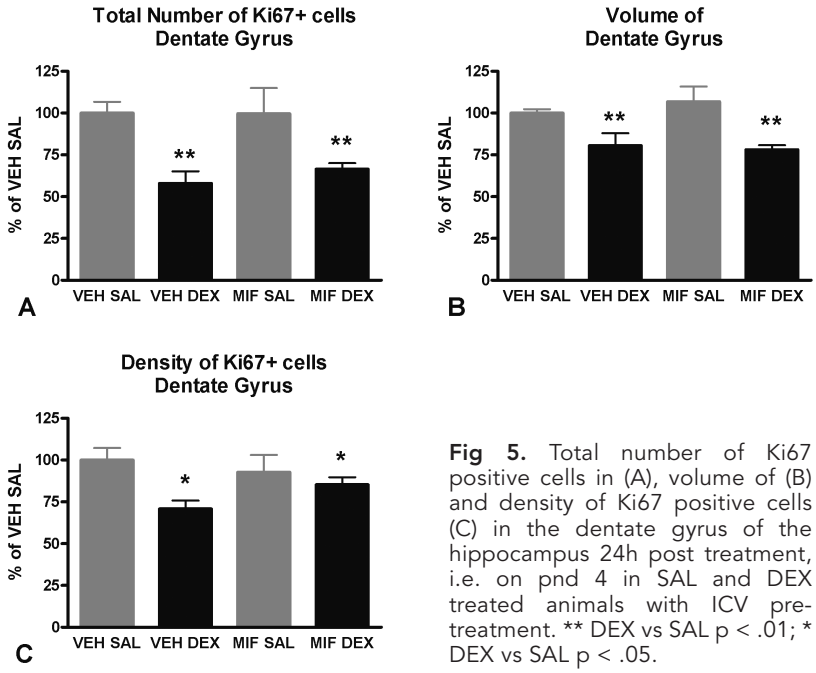

Fig 5. Total number of Ki67 positive cells in (A), volume of (B) and density of Ki67 positive cells (C) in the dentate gyrus of the hippocampus 24h post treatment, i.e. on pnd 4 in SAL and DEX treated animals with ICV pre-treatment. ** DEX vs SAL $p < .01$; * DEX vs SAL $p < .05$.

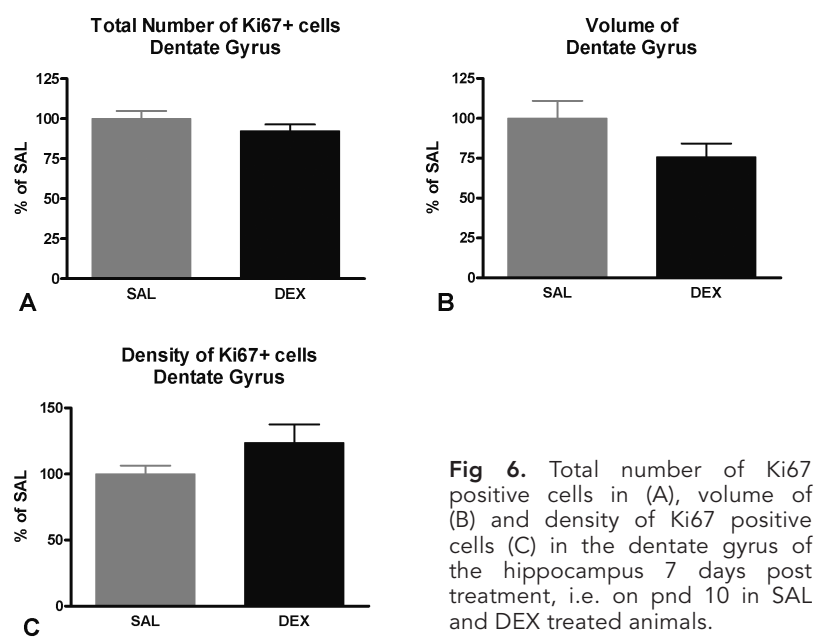

Fig 6. Total number of Ki67 positive cells in (A), volume of (B) and density of Ki67 positive cells (C) in the dentate gyrus of the hippocampus 7 days post treatment, i.e. on pnd 10 in SAL and DEX treated animals.

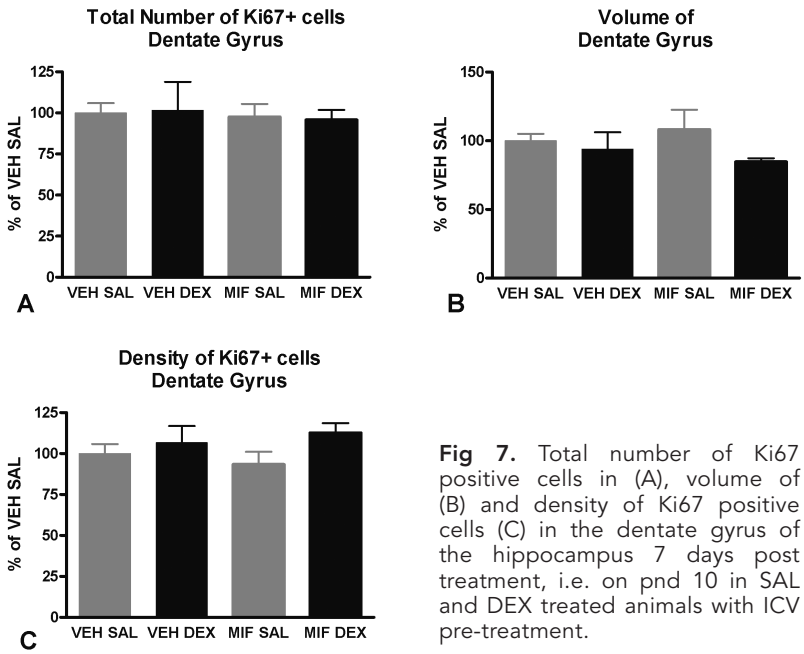

Fig 7. Total number of Ki67 positive cells in (A), volume of (B) and density of Ki67 positive cells (C) in the dentate gyrus of the hippocampus 7 days post treatment, i.e. on pnd 10 in SAL and DEX treated animals with ICV pre-treatment.

DISCUSSION

We report that neonatal DEX treatment had differential effects on GFAP expression 24h and 7 days post treatment which could be (partially) prevented by central GR antagonist pre-treatment. Additionally, we report that neonatal DEX treatment acutely reduced DG cell proliferation and volume. These effects had normalized 1 week post treatment, and were not affected by central GR antagonist pre-treatment. These findings indicate that neonatal DEX treatment exerts acute and delayed as well as transient effects on the developing brain. Additionally, central anti-glucocorticoid pre-treatment may serve as a potential early intervention strategy, partially normalizing DEX-induced changes in the developing brain.

Differential effects of DEX on glial number and activity

The effect of DEX on GFAP expression was two-fold. First, the *density* of GFAP expression tended to be acutely reduced in the CC by DEX treatment in animals that received ICV injections, whereas DEX treatment did not acutely affect GFAP expression in non-ICV injected animals. Interestingly, the overall expression of GFAP was higher in the ICV injected groups as compared to the non-ICV injected groups (data not shown). A diffuse glial scar comprising reactive astrocytes is likely to form in response to 3 consecutive days of bilateral ICV injections (20, 21). This reactive gliosis tended to be responsive to DEX treatment. GCs have been reported before to regulate the number of GFAP positive astrocytes (22), the production of GFAP in astrocytes (23) and their differentiation (24). Additionally,

steroid hormones have been shown to reduce reactive gliosis upon injury (25, 26). Since the glial scar has characteristics leading to inhibition of neuronal growth and regeneration (27), strategies to reduce these characteristics are used as treatment for brain injury (28). It has been reported that the gliosis-reducing effect of steroid hormones is associated with enhanced functional recovery after injury (25) and can therefore be considered a beneficial effect.

Secondly, DEX treatment led to a significant reduction in the *number* of GFAP positive cells 7 days post treatment, both in animals with and without ICV treatment, which could be fully restored by central GR antagonist pre-treatment. Several studies have shown GC-induced reductions in GFAP expression both in terms of RNA or protein levels (23, 29, 30) and number of GFAP positive cells (22). Unfortunately we were not able to quantify cell numbers on pnd 4. Therefore we cannot rule out whether an acute DEX-induced reduction in glial cell number on day 4 was maintained until day 10, or whether this effect developed progressively during maturation of the brain.

Whereas the gliosis-suppressing effect of DEX could have a beneficial effect, the reduction in cell number, present in all DEX treated animals, appears detrimental considering the supportive role of astrocytes in the brain especially during development (31). Huang and colleagues have reported a delayed maturation of astrocytes in CC after GC treatment, leading to a delayed tightening of the blood-brain barrier, which is suggested to have detrimental effects on CNS development (30). Additionally, GC exposure leads to delayed myelination of callosal fibers (32) and disturbances in long-term maintenance of myelin (33). These disturbances led to decreased conduction velocity potentially affecting information processing and cognitive performance (34). A reduction in astrocyte number during development is likely to mediate and/or contribute to this phenomenon and might underlie the frequently reported cognitive impairments after neonatal synthetic GC exposure (7, 12).

DEX temporarily suppresses hippocampal cell proliferation

We report that neonatal exposure to DEX acutely reduced DG cell proliferation, an effect that had normalized by pnd 10. The inhibitory effect of this GC on cell proliferation has been shown frequently, with some studies reporting a transient nature (35, 36) and others showing lasting effects of early GC or stress treatment (14, 37). The current findings are in line with previous findings from Tauber and colleagues showing that early exposure to DEX resulted in reduced cell proliferation in the newborn marmoset (38) which was restored to control levels at 2 year of age (39) indicating that the decreased proliferation rate in the newborn is transient.

From these, and the current findings it should not be concluded that perinatal GC treatment does not have long-lasting consequences and that its use can be considered safe. In rodents, the development of the hippocampus, especially the DG, happens largely during the early postnatal period (40-43). It is believed that the extensive plasticity that characterizes normal early development is necessary to

set the stage for proper adult functioning of the hippocampus, especially in terms of adult neurogenesis (43-45). This indicates that, even a temporary reduction in cell proliferation during a developmental stage that is normally characterized by high levels of proliferation, can exert long-lasting effects on the functioning of the hippocampus and other connected brain structures. These alterations could contribute to the frequently reported cognitive impairments in both animals and humans neonatally exposed to synthetic GCs (7, 12).

Mifepristone and anti-progesterone activity in proliferation

One of the aims of this study was to investigate the possibility to prevent DEX-induced alterations in the developing brain by blocking the GR during DEX treatment. We report that the DEX-induced reduction in glial cell number observed on pnd 10 was fully normalized by central mifepristone pre-treatment. However, the substantial reduction in hippocampal cell proliferation, observed on pnd 4 was not prevented by mifepristone pre-treatment. Blocking the GC-induced reduction in cell proliferation by mifepristone was expected to induce a proliferation-enhancing effect (14, 35, 38, 46) by promoting the protective function of the mineralocorticoid receptor (MR) (47). However, we did not observe this effect in the current study.

The role of mifepristone in cell proliferation is however multidimensional. Besides blocking GR mediated effects and enhancing the role of MR (altogether enhancing proliferation), mifepristone also has intrinsic anti-proliferative effects (48). These effects are likely the result of its anti-progesterone activity (49). Since we did not observe anti-proliferative effects in the MIF SAL animals or investigate the role of progesterone activity in this experiment, we cannot conclude that progesterone receptor-mediated effects overruled those mediated via GR. Altogether, these findings suggest that in future studies, the possibility of blocking DEX-induced, GR-dependent effects should be investigated using a selective GR antagonist.

Factors mediating function of GR ligands

There are additional factors that mediate the outcome of treatment with GR ligands. Under certain conditions mifepristone can exert effects similar to those induced by DEX (50) and act as a full GR agonist with respect to GR-mediated transrepression (51). Therefore, DEX-induced alterations depending on GR-mediated transrepression are less prone to be prevented by mifepristone pre-treatment.

Additionally, alterations in GR density determine the degree of (ant)agonistic activity of GR ligands in transrepression as well as the efficacy and potency of DEX (52). GR expression is known to be affected by neonatal GC exposure (53) and since these effects appear rather acute (within days) (54), differences in central GR density between the treatment groups may develop during the 3 days of drug treatment. This variation might lead to group-specific alterations in the behaviour of the GR ligands (both agonist and antagonist) in terms of GR-mediated transrepression.

MR mediated effects

The finding that not all DEX-induced effects can be normalized using GR antagonist pre-treatment could also indicate that DEX exerts it function partially via another receptor or mechanism. For example, it has been suggested that DEX, despite a substantially higher affinity for GR, binds to MR as well (55-57). Since the blood-brain barrier is not fully developed in the neonate (58, 59) GCs may readily enter the brain (60, 61) at these life stages, where they may be able to target central MR in addition to GR, which are both expressed in the developing brain (61, 62). Certain DEX-induced alterations can be counteracted if there is sufficient MR activation (63).

The daily procedure of removing pups from the nest followed by handling and injections can be considered an experience disrupting the neonate's stress hyporesponsive period, potentially leading to an inappropriate rise in endogenous GC levels (64, 65). This potential rise in corticosterone can be modulated by DEX (66) and mifepristone leading to a complex interplay between endogenous and exogenous (anti-) glucocorticoids, resulting in differential MR activation profiles in the different experimental groups.

CONCLUDING REMARKS

The aim of this study was to investigate the possibility to block DEX-induced changes on the developing brain using GR antagonist pre-treatment. To summarize, DEX treatment exerts acute, delayed as well as transient effects, which might contribute to the frequently reported long-term functional alterations reported following neonatal GC treatment. DEX-induced alterations can be partially prevented by central mifepristone pre-treatment. Therefore we suggest that these findings represent a *proof of principle* for the use of central GR antagonist pre-treatment as a potential intervention strategy to block DEX-induced alterations. It has to be noted however, that there is only partial normalization and that the route of mifepristone administration is relatively invasive, resulting in an additional impact (reactive gliosis) on the developing brain. Future studies are therefore needed to investigate alternative routes of administration (such as intrathecal therapy) to determine the clinical potential of this intervention strategy.

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CHAPTER 4

Developmental and long-lasting consequences of neonatal dexamethasone treatment: impact of early handling

Sanne E.F. Claessens, Melly S. Oitzl,
Nikolaos P. Daskalakis and E. Ronald de Kloet

Division of Medical Pharmacology, LACDR/LUMC, Leiden University, The Netherlands

Submitted

ABSTRACT

Exposure to increased glucocorticoid levels during development is known to have enduring consequences for brain function and behaviour throughout the lifespan. Perinatal dexamethasone administration, a common treatment for prematurity-associated respiratory disorders and an example of such enhanced glucocorticoid exposure, has been shown to result in adverse side effects on the developing nervous system, leading to long-lasting alterations in endocrine and behavioural phenotype.

In the current study we investigated the development of these alterations in male Long Evans rats. Rat pups were injected with dexamethasone or saline on postnatal days 1, 2 and 3. In experiment I, body weight, eye opening and fur development were measured during the postnatal period. In adulthood animals were tested for spatial learning, stress responsiveness, and contextual fear conditioning.

Dexamethasone treatment resulted in growth retardation, an altered pattern of fur development and accelerated eye opening. However, we did not observe alterations in behavioural and endocrine phenotype in adulthood. Therefore, we investigated a potential interaction of the effects of dexamethasone treatment with those of neonatal handling, which was an inevitable component of our experimental design. Thus, we included in experiment II an untreated non-handled control group. We report that these non-handled controls displayed reduced pre-pulse inhibition, motor performance and spatial learning in addition to a prolonged endocrine stress response, compared to handled animals (both saline and dexamethasone-treated).

We conclude that neonatal handling resulted in profound phenotypic alterations throughout the lifespan, potentially protecting against dexamethasone-induced alterations.

INTRODUCTION

Perinatal life represents a critical period during which an individual is highly susceptible to environmental influences. Adverse infant experiences have been shown to induce profound and long-lasting effects on adult brain function and behaviour (1, 2) that may increase vulnerability for disease development (3-5). These enduring effects of early experiences may be associated with a long-lasting impact on the functioning of the hypothalamic-pituitary-adrenal (HPA) axis (3, 4, 6-8).

During normal early development, the brain appears to be protected from exposure to high levels of endogenous glucocorticoids, since the neonate displays a strongly reduced adrenocortical response to mild stressors, a phenomenon reported in rodents (9-11) and humans (6). This stress hypo-responsive period (SHRP) can only be disrupted when the organism is either exposed to extremely stressful (life threatening) events or when the caregiver is absent. In absence of the mother, corticosterone levels slowly increase and the neonate becomes responsive to mild stressors that would not result in HPA axis activation in her presence (11-13). Emergence from the SHRP, and subsequent exposure to elevated levels of glucocorticoids has profound programming effects on development and is important for shaping the adult phenotype (14-16).

Exposure to exogenous glucocorticoids such as dexamethasone (DEX) can be considered a model for inappropriate glucocorticoid secretion. Interestingly, DEX administration is a common treatment for prematurity-associated respiratory disorders and supposedly a life saving treatment (17). However, as can be expected from neonatal glucocorticoid exposure, this treatment has profound side effects on the developing nervous system, leading to long-lasting alterations in brain function and behaviour both in humans (18) and animals (19). DEX-treated rodents show spatial learning impairments, altered endocrine responsiveness to acute stress and a significantly shortened lifespan (19-21).

In the current study we investigated the development of these alterations in cognitive performance and endocrine stress responsiveness after neonatal glucocorticoid treatment in a rodent model, which is described in experiment I. Interestingly we did not observe the previously reported detrimental effects of neonatal glucocorticoid exposure on adult phenotype. We suggest that neonatal handling, as an inevitable component of the experimental design, might serve as a factor protecting against the adverse effects of neonatal glucocorticoid exposure. This hypothesis was tested in experiment II, using untreated non-handled animals as an additional control group.

MATERIALS AND METHODS

Animals

Adult Long Evans rats from our breeding population were used as breeders. Two females were mated with one male for 10 days in type 4 polycarbonate cages (59x38x20cm) containing sawdust bedding and tissues. Food (RM3, Special Diet

Services, Witham, Essex, UK) and water (8 ml 25% HCl /10 L tap water) were provided *ad libitum*. Animals were maintained on a 11-h light : 13-h dark cycle with lights on at 08.30h, in a temperature (21± 2°C) and humidity (55 ± 5%) controlled room. After breeding, pregnant females were individually housed. Females were checked daily for presence of pups. If pups were present, the day of birth for that particular litter was defined as postnatal day 0 (pnd 0). On pnd 1, litters were culled to 8 pups (4 male and 4 female). Cages were cleaned at pnd 10 and after weaning once weekly. Animal experiments were approved by the Local Committee for Animal Health, Ethics and Research of Leiden University and carried out in accordance with European Communities Council Directive (86/609/EEC).

Experimental Design

Experiment I: To investigate the consequences of neonatal DEX treatment on development and adult phenotype we studied saline (SAL) and DEX treated offspring according to the schedule depicted in fig. 1.

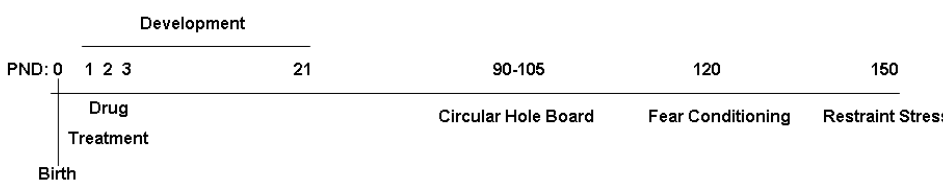

Fig 1. Timeline experiment I. PND: postnatal day.

Experiment II: To investigate whether the effects of neonatal handling, induced by daily marking and weighing of the offspring, might have protected against DEX-induced alterations, we extended the experiment with an untreated non-handled (UNT NH) control group. Animals from litters born at the same time in the same breeding colony as the SAL and DEX animals, were included for this purpose at the age of 6 months. These animals were raised and housed under similar conditions as the SAL H and DEX H animals, except for drug administration and daily handling during the postnatal period. They were not subjected to the two behavioural tests in experiment I, and thus kept undisturbed except for a weekly cage cleaning. SAL and DEX treated animals will, in experiment II, be referred to as SAL H and DEX H. Animals were tested according to the schedule depicted in fig. 2.

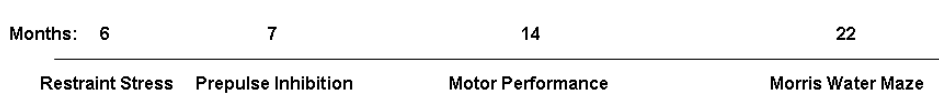

Fig 2. Timeline experiment II.

Drug Treatment

Male pups were randomly assigned to either the saline (SAL) or the dexamethasone (DEX) group using a within litter design. Pups in the DEX group were subcutaneously (SC) injected with dexamethasone-21-phosphate (Sigma Aldrich, Zwijndrecht, The Netherlands) on pnd 1 (0,5 µg/g body weight), pnd 2 (0,3 µg/g) and pnd 3 (0,1 µg/g). Pups in the SAL group were injected with equivalent volumes of sterile and pyrogen free saline. In order to prevent bias for the dam to show enhanced attention for injected or non-injected offspring, all female littermates were injected with SAL on pnd 1, 2 and 3. All pups were daily marked using a non-toxic, odourless marker in order to discriminate between littermates assigned to either the SAL or DEX group.

Postnatal development

Postnatal development was monitored in SAL and DEX treated pups. Body weight, eye opening and fur development were recorded. For eye opening a scale from 0-9 was used indicating: 0 = closed, 3 = occasional/partial opening, 6 = mostly open, 9 = fully open. For fur development a scale from 0-12 was used indicating: 0 = no fur, 3 = fine hairs, 6 = partial fur, 9 = mostly fur, 12 = full fur (adapted from (22)).

General procedure

Pnd 1-3: The daily procedure consisted of removing all pups from the nest followed by transfer to an adjacent room, where the holding cage was put on a heating pad. Body weights (BW), eye opening and status of fur development were recorded, followed by marking and injection of the pups. After the procedure (which took ~ 10 minutes per litter) all pups were immediately returned to the home cage.

Pnd 4-21: The daily procedure consisted of removing all pups from the nest followed by transfer to an adjacent room, where the holding cage was put on a heating pad. Body weights, eye opening and status of fur development were recorded, followed by marking of the pups. After the procedure (which took ~ 6 minutes per litter) all pups were immediately returned to the home cage.

Besides these manipulations (which always took place between 9:00 and 13:00h) litters were left undisturbed until weaning on pnd 22, except for a cage change on pnd 10.

Adult Phenotype

Spatial learning: Circular Hole Board

Spatial learning was assessed between pnd 90 - 105. The Circular Hole Board consisted of a white circular platform (120 cm diameter) with 12 holes (10 cm diameter) equidistantly placed at 12 cm from the edge of the platform. An overhead camera allowed tracking of the behaviour of the animals during testing. Distal spatial cues were mounted on the walls of the testing room for orientation. The paradigm is based on the assumption that rodents are motivated to find a way to escape when being exposed to a light and unprotected place like the hole board. The animals were trained to locate the position of the exit hole that

leads via an escape tunnel to the home cage. All other holes were closed with a lid placed 1 cm below the surface of the platform. This prevented the animal from seeing whether a hole is open or closed until it is in close proximity to the hole. The exit hole was always in the same spatial location; however, the platform was turned between trials to eliminate odour cues. All trials started by putting the animal in a start tube (diameter: 20 cm, height: 30 cm) that was positioned in the centre of the platform and removed to start the trial. If an animal did not find the exit hole within 120 seconds, it was gently guided there by the investigator. The platform was cleaned in between sessions using a 10% ethanol solution to eliminate odour cues. All testing took place between 10.00 and 13.00h.

General procedure

In the week prior to hole board training, animals were exposed to 3 sessions of tunnel training to practice manoeuvring through the escape tunnel that will lead to the home cage during hole board training.

On day 1, to familiarize the animals with the platform and the existence of an exit hole plus escape tunnel, animals were allowed to explore the platform during a 3 min free exploration trial (FET) with all holes closed. At the end of the 3 min period, the exit hole was opened and the animal was given another 2 min to locate the exit hole.

On day 2, animals were trained during 6 trials with a 15 min inter-trial interval to find the location of the exit hole.

On day 8, animals were retested to evaluate spatial memory during 2 memory trials, using similar conditions as during training on day 2.

Total distance moved, amount of time spend in different areas/quadrants of the platform, number of holes visited, latency to find exit hole and latency to escape were analyzed using EthoVision XT (Noldus Information Technology, Wageningen, The Netherlands).

Contextual Fear Conditioning

Contextual fear memory was assessed between pnd 120-130. The shock box (40x40x50 cm) was made of black Plexiglas walls and a stainless steel rod floor, connected to a shock generator. The box was cleaned with a 10% ethanol solution between all sessions to eliminate odour cues. An overhead video camera recorded behaviour of the animals throughout all sessions. All testing took place between 10.00 and 13.00h.

General procedure

On day 1 the animal was transferred from the housing room to the adjacent test room where it was placed in the shock box. After 2 min it was exposed to a foot shock of 0.6 mA (duration 2 sec). After the shock the animal remained in the shock box for 2 minutes and was then removed from the box and transferred back to the home cage. 24 hours later (day 2), the animal was re-exposed to the shock box for 4 min, however without receiving a foot shock.

Behaviour of the animal was recorded both during training and re-exposure and analyzed by an observer unaware of treatment conditions using The Observer

9.0 XT (Noldus Information Technology, Wageningen, The Netherlands). The following behaviours were scored (1) freezing (lack of all body movement except those necessary for breathing), (2) scanning (lack of movements, except lateral head movements and movements necessary for breathing), (3) rearing, (4) walking, and (5) sitting. Behaviour was analyzed during 3 distinct time periods: (1) 2 min before shock, (2) 2 min after shock, (3) 4 min re-exposure.

Endocrine response to restraint stress

On pnd 150, endocrine HPA axis activation in response to an acute restraint stressor was tested. One day prior to restraint stress a basal blood sample was taken from the tail vein. The next day animals were exposed to 10 min of restraint stress by placing them in a custom made restrainer, which restricts body movements. Blood samples were taken at 2, 5, 10 30, 60, and 120 min.

Hormone Analysis

Blood samples were collected in EDTA coated tubes (Microvette CB 300 K2E, Sarstedt, Germany). Samples were kept on ice and centrifuged for 15 min at 13000 rpm at 4°C. Plasma was transferred to Eppendorf tubes and stored at -20°C until further analysis. Plasma adrenocorticotropic hormone (ACTH) and corticosterone (CORT) concentration were measured using a commercially available radio immuno assay (RIA) kit containing ^{125}Iodine labelled ACTH or CORT, respectively (MP Biomedicals Inc., USA). All samples were processed in the same assay to exclude inter-assay variability.

Prepulse Inhibition

Prepulse inhibition was measured at 7 months of age. Three/four littermates were transferred together to the test room where they were allowed to habituate for 45 min. After habituation they were placed in a startle recording apparatus (SR-LAB, San Diego Instruments, CA, USA), containing a transparent Plexiglas tube (diameter 8.7 cm, length 20.5 cm) mounted on a Plexiglas base. Sounds were presented by a speaker and movement of the animals was detected by a piezoelectric accelerometer mounted below the Plexiglas tube and recorded by a computer. Testing started with a five min habituation session with background white noise of 70 dB[A]. Animals were first presented with six pulse alone trials (117 dB[A]) followed by 39 trials comprising different trial types according to a pseudo-randomized schedule with an inter-trial interval of 10-20 sec. Trial types: 4x background white noise alone, 5x prepulse alone (16 dB[A] above background = 86 dB[A]), 20x prepulse–pulse trials using prepulse intensities of 2, 4, 8, 16 dB[A] above background noise (i.e. 72, 74, 78 and 86 dB[A]), and 10x pulse alone (117 dB[A]). Finally, animals were again exposed to five pulse alone trials. The duration of the prepulses was 20 ms, duration of the pulses was 40 ms. Prepulse to pulse interval was 100 ms. Startle activity was measured during 100 ms after onset of the pulse. The percentage PPI at the different prepulse intensities was calculated as [100-(100 x startle amplitude at prepulse trial)/ (startle amplitude at

startle pulse-alone trial)]. Speakers were calibrated every day. Experiments were performed between 9:00 and 13:00 to minimize circadian influence.

Motor performance

At 14 months of age all animals were tested in a motor performance test battery. Testing took place over 4 different test sessions on 2 consecutive days.

Balance beam

The animal was placed on a square metal bridge (2 cm x 2 cm x 40 cm, elevated about 40 cm above the surface) wrapped in anti slip tape. The duration the animal stayed on the bridge was measured to a maximum of 120 s. A pillow was placed beneath the bridge to cushion the animal's fall. Rats were tested twice a day on 2 consecutive days.

Rota-rod

Motor coordination was measured using the rota-rod test. In this test, the animal was placed on the rota-rod treadmill, attached to a rotating motor. The treadmill consisted of four rotating drums (7 cm diameter, 24 cm above ground), divided by flanges. The first day, rats were familiarized with the apparatus. During 2 habituation trials - one in the morning and one in the afternoon - which lasted 2 min each, the animals were placed on the constantly revolving drum (speed 13 rpm). If a rat fell off during habituation it was placed back immediately. Number of falls during the 2 min habituation periods was recorded. On the second testing day, the animals were tested on an accelerating rota-rod (up to 40 rpm in max 3 min). Latency to fall from the rotating rod was recorded.

Foot-fault test

This test measures placement dysfunction of the paws and motor coordination. The animals were placed on an elevated wire grid (dimension 100 x 100 cm, grid size 4 x 9 cm) and allowed to explore the grid for 2 minutes. Number of steps and number of errors (foot-faults were counted when a paw fell completely through the bars of the grid) were recorded.

Water maze performance

At 22 months of age spatial learning in a water maze was tested. Animals were placed, without prior training, in a pool (150 cm diameter) filled with 30 cm of water (21°C) made opaque by adding 3 spoons of latex paint. A platform (10 cm diameter) was hidden 1 cm below the surface of the water and was positioned in the NE quadrant.

Animals were given 2 daily trials (inter-trial interval 15 min) to find the platform on day 1, 2 and 3. On day 4, one more training trial was given, followed by a probe trial without platform present. On day 5, animals were given 3 reversal trials with the platform located in the position opposite (SW) from where it was during training. Trials always started in one of the 3 quadrants where the platform was not located in a pseudo-randomized fashion, following the same order of start position for every animal. If an animal did not reach the platform within 2 minutes,

it was gently guided there by the investigator. Animals were allowed to stay on the platform for 15 sec after finding it or being guided there.

Swim patterns were tracked by an overhead camera and later analysed using EthoVision XT (Noldus Information Technology, Wageningen, The Netherlands). Frequencies and time spent in target and other quadrants, or in close proximity to the platform (or the location where the platform used to be) were analyzed during training and reversal trials, as well as swim patterns during probe trials. After every swim trial, animals were dried with a towel and kept in a clean cage lined with clean dry tissues, placed on a heating pad. All trials took place between 10.00 and 13.00h.

Survival

Survival of the animals was determined by recording the health span rather than the lifespan. If an animal died without showing signs of pathology, the age of natural death was recorded. However, if an animal did show signs of pathology (i.e. substantial weight loss, impaired locomotion, breathing problems) the animal was sacrificed and age of sacrifice was recorded.

Data analysis

Data are presented as mean +/- SEM. All data were analysed using repeated measures ANOVA with time (age, trial, day or min) as within and drug treatment and handling as between subject factors, one-way ANOVA, or paired t-test with level of significance set at $p < .05$. Where appropriate main effects and interactions were further investigated using post-hoc analysis with appropriate correction for multiple testing. Corrections for violations of sphericity were conducted where needed. SAL H and DEX H animals were always initially included as separate groups in the analysis. If drug treatment did not significantly contribute to the outcome, data from SAL H and DEX H animals were pooled to investigate the impact of neonatal handling by comparing performance of these 2 groups to UNT NH animals.

RESULTS EXPERIMENT I

Postnatal development

Body weight gain is reduced in DEX treated animals during the postnatal period (fig. 3A) and remains lower throughout adult life (fig. 3B) (main effect of drug treatment $F(1,18) = 26.01$, $p < .001$). A significant time x drug treatment interaction was observed ($F(36,648) = 5.62$, $p < .001$). Post-hoc analysis per time point revealed a significant difference between SAL and DEX on all time points starting at pnd 2 ($p < .001$ until week 10, $p < .01$ until week 24, and $p < .05$ at week 26).

Neonatal DEX treatment results in accelerated eye opening (fig. 4A) and an altered pattern of postnatal fur development (fig. 4B). Besides a significant main effect of time ($F(20,520) = 1382.18$, $p < .001$) and drug treatment ($F(1,26) = 168.12$,

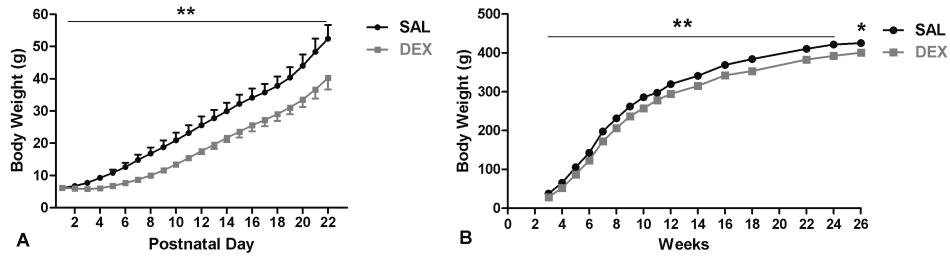

Fig 3. Body weight of SAL and DEX treated animals during the postnatal (A) and post-weaning (B) period. DEX treated animals show significantly lower body weight compared to SAL treated animals at all time points starting on pnd 2. ** $p < .01$; * $p < .05$

$p < .001$) a significant time x drug treatment interaction ($F(20,520) = 56.81$, $p < .001$) was observed for eye opening. Post-hoc analysis per time point revealed significant differences between SAL and DEX treated animals on pnd 10, 11, 12 and 13 indicating that DEX treated animals open their eyes at an earlier age.

For fur development we observed, besides significant main effects of time ($F(20,500) = 2059.70$, $p < .001$) and drug treatment ($F(1,25) = 27.70$, $p < .001$), a significant time x drug treatment interaction ($F(20,500) = 40.36$, $p < .001$). Post-hoc analysis per time point revealed that DEX treated animals score significantly higher on pnd 6, 7 and 8, whereas SAL treated animals score significantly higher on pnd 11-20 (all $< .01$ and pnd 20 $p = .044$) leading to full fur development 4 days earlier in SAL compared to DEX treated animals.

Spatial learning: Circular Hole Board

We analyzed behaviour during all 6 trials and performance during trial 1 and 2 separately. For latency to first visit the exit hole (fig. 5A) and latency to escape

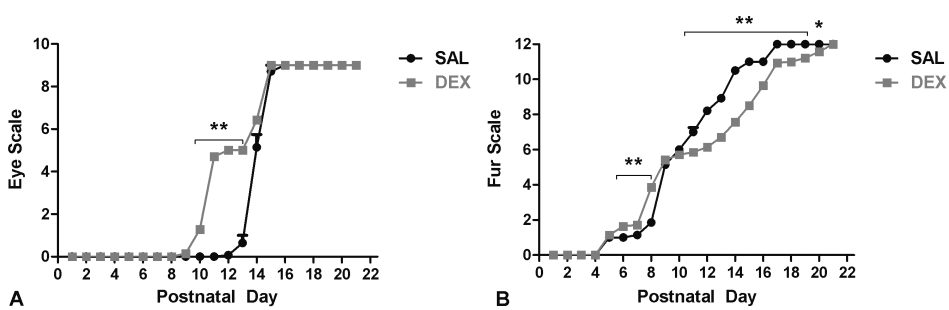

Fig 4. Timing of eye opening (A) and fur development (B) in SAL and DEX treated animals during the postnatal period. DEX treated animals show, compared to SAL treated animals, accelerated eye opening and a temporary advantage in fur development. However by pnd 11 SAL treated animals catch up leading eventually to full fur development 4 days earlier compared to DEX treated animals. ** $p < .01$, * $p < .05$

(fig. 5B), significant main effects of time were observed, ($F(2.98,62.47) = 3.08$, $p = .034$) and ($F(3.62,76.09) = 6.60$, $p < .001$) respectively. No effect of drug treatment was observed. For number of holes visited (before the first visit to exit hole, fig. 5C) performance tended to be different between SAL and DEX treated animals ($F(1,21) = 3.28$, $p = .084$). When trial 1 and 2 were analyzed separately, a significant main effect of drug treatment ($F(1,21) = 4.64$, $p = .043$) was observed indicating that DEX treated animals visit significantly fewer holes before going to the one that leads to the escape tunnel, compared to SAL treated animals. Additionally, when the percentage of animals visiting the correct quadrant of the platform during their first hole (any) visit was investigated, we observed that a slightly higher (although not statistically significant) percentage of DEX treated animals search for the exit hole in the correct quadrant compared to SAL treated animals in trial 1 (χ^2 (1) 3.486, $p = .062$; fig. 5D). Behaviour during the memory trial was not different between SAL and DEX treated animals.

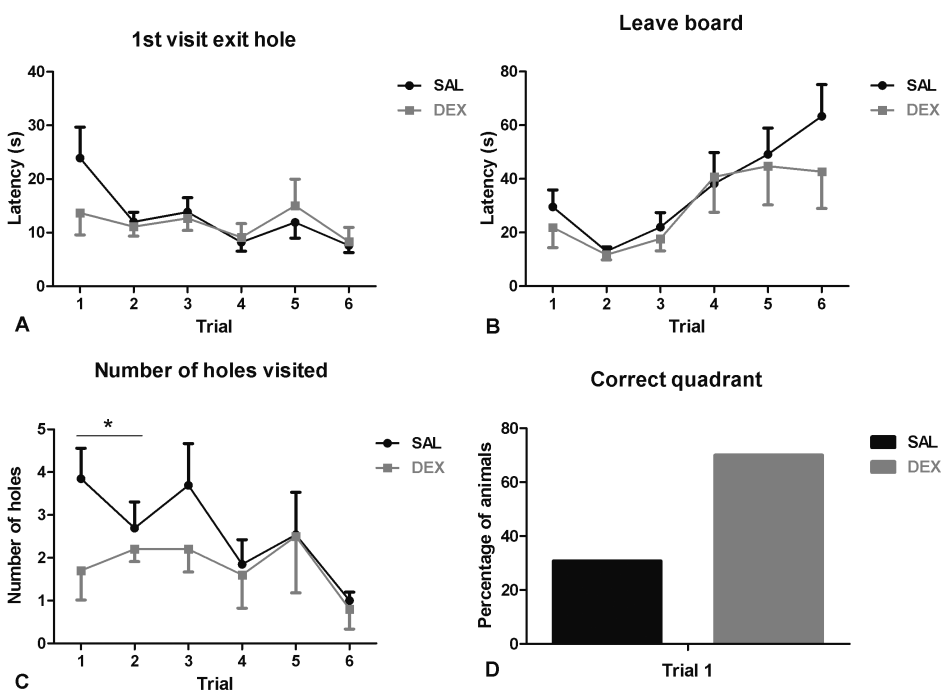

Fig 5. Spatial learning on the Circular Hole Board. Latency to first visit the exit hole (A) and latency to leave the platform via exit hole (B) is not different between SAL and DEX treated animals. DEX treated animals visit fewer holes before finding the exit hole compared to SAL treated animals during the first 2 training trails (C). A higher percentage of DEX treated animals visits the correct quadrant of the hole board when visiting a hole for the first time compared to SAL treated animals, although this effect is not statistically significant (D). * $p < .05$.

Contextual fear conditioning

Freezing, as well as other behaviours measured in the fear conditioning paradigm were not significantly affected by neonatal exposure to glucocorticoids (data not shown).

Endocrine response to restraint stress

We did not observe DEX-induced changes in ACTH or CORT level under basal conditions or in response to acute restraint stress in adulthood (data not shown).

RESULTS EXPERIMENT II

Endocrine response to restraint stress

Since basal and restraint stress-induced ACTH and CORT levels were not different between SAL H and DEX H animals, data from these groups were pooled and analyzed as a handling (H) group. For ACTH a significant effect of time ($F(2.76,63.46) = 62.59$, $p < .001$) and a time x handling interaction ($F(2.76,63.46) = 3.44$, $p = .025$) were observed. The effect of handling did not reach statistical significance ($F(1,23) = 4.06$, $p = .056$).

Post-hoc analysis per time point revealed a significantly lower ACTH level in H animals at t2 ($p = .044$), t5 ($p = .002$), t60 ($p = .048$) and t120 ($p = .000$), while peak level (t30) was not different (fig. 6A). Basal ACTH levels appeared to be higher H animals, although this effect was not statistically significant ($p = .051$).

For CORT level, a significant main effect of time ($F(3.03,87.78) = 331.21$, $p < .001$) and a time x handling interaction ($F(3.03,87.78) = 10.32$, $p < .001$) were observed. Post-hoc analysis per time point indicates that H animals have significantly higher CORT at t30 ($p = .006$) but lower CORT at t120 ($p < .001$) (fig. 6B).

Prepulse Inhibition

Mean PPI is significantly different between UNT NH animals and animals from the two H groups (SAL H and DEX H) ($F(2,23) = 22.01$, $p < .001$) indicating that

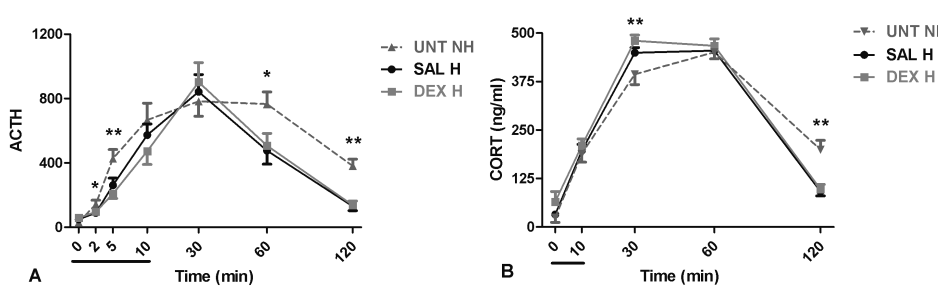

Fig 6. Plasma ACTH (A) and CORT (B) levels before, during and after 10 min restraint stress (indicated with horizontal black bar). H animals displayed lower ACTH levels at t2, t5, t60 and t120 compared to NH animals. H animals show higher CORT levels at t30 but lower CORT levels at t120. ** $p < .01$, * $p < .05$

H animals show enhanced PPI compared to UNT NH animals. Post-hoc analysis revealed significant differences between UNT and SAL ($p < .001$) and between UNT and DEX ($p < .001$) (fig. 7).

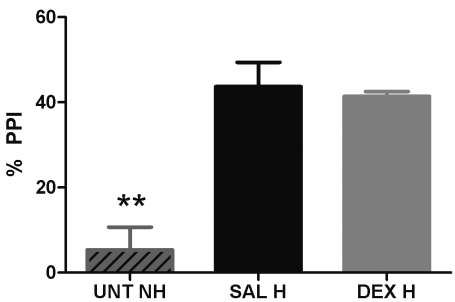

Fig 7. Prepulse Inhibition. H animals (SAL H and DEX H) show enhanced PPI compared to UNT NH animals. ** $p < .01$

Motor performance

Balance beam

Performance (latency to fall) did not differ between treatment groups when analyzed per session. When performance was analyzed within groups to investigate improvement over test sessions, repeated measures ANOVA revealed no improvement in UNT NH animals (fig. 8A). Since SAL H and DEX H did not show differences in performance, data from these groups were pooled. A significant main effect of time was observed ($F(1.67,35.07) = 4.35$, $p = .026$) indicating that performance improved over the different test sessions in H animals (fig. 8B). Moreover a paired t-test showed that performance on day 2 was significantly higher compared to day 1 in H animals ($t = -2.362$ (21), $p = .028$), whereas there was no such effect in UNT NH animals.

Rota-rod

Overall one-way ANOVA revealed that performance during habituation session 1 is comparable for all groups (fig. 9A). During habituation session 2 however, UNT NH animals fall significantly more compared to the two H groups ($F(2,30) = 3.45$, $p = .045$, fig. 9B). All groups fall less frequently during the 2nd compared to the 1st habituation session, however this finding indicates that improvement is stronger in H animals.

Performance on the accelerating rota-rod - on test day 2 - revealed that UNT NH appear to have a shorter latency to fall compared to H animals, although this effect was not statistically significant ($F(2,30) = 3.23$, $p = .054$; fig. 9C).

Foot-fault test

One-way ANOVA indicated that the total number of steps was somewhat lower (although not statistically significant) in DEX H compared to SAL H and UNT NH animals ($F(2,29) = 2.97$, $p = .067$, fig. 10). The error rate was not different between groups (26-27% in all groups).

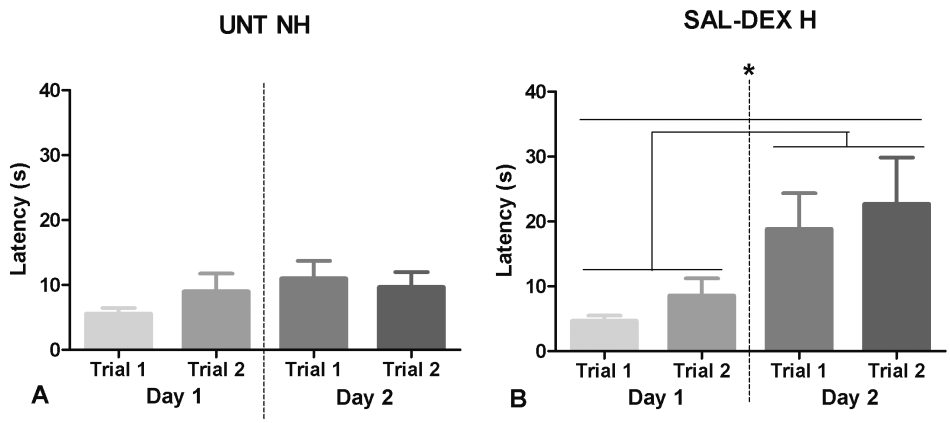

Fig 8. Latency to fall from balance beam during 4 sessions over 2 test days. UNT NH (A) animals do not show improvement (increased latency to fall) in contrast to H animals (B) which improve significantly during the different sessions. * $p < .05$

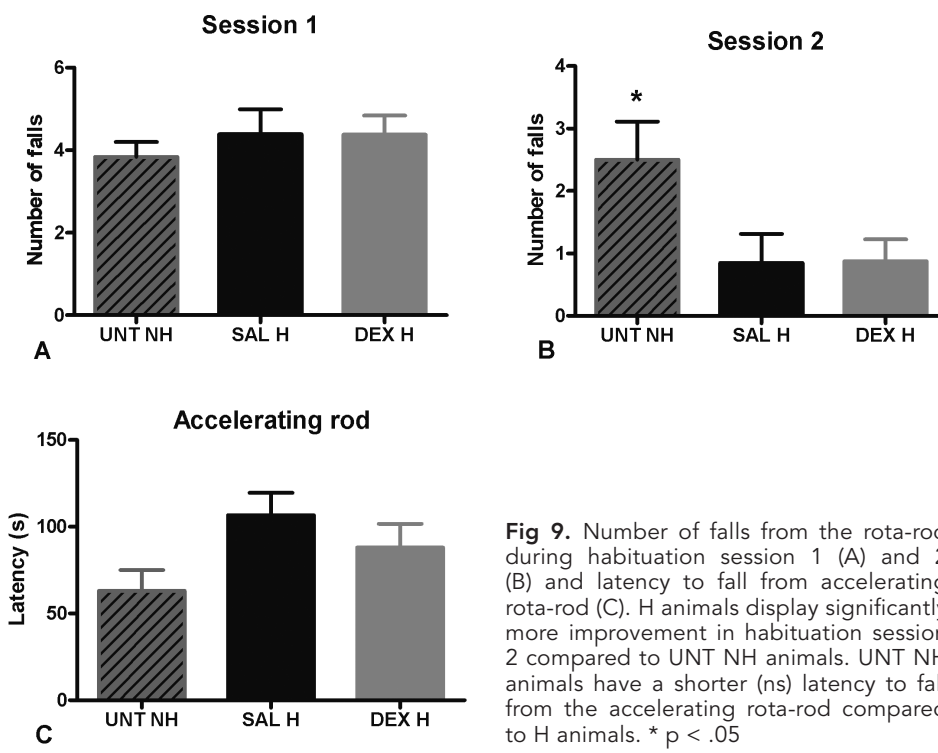

Fig 9. Number of falls from the rota-rod during habituation session 1 (A) and 2 (B) and latency to fall from accelerating rota-rod (C). H animals display significantly more improvement in habituation session 2 compared to UNT NH animals. UNT NH animals have a shorter (ns) latency to fall from the accelerating rota-rod compared to H animals. * $p < .05$

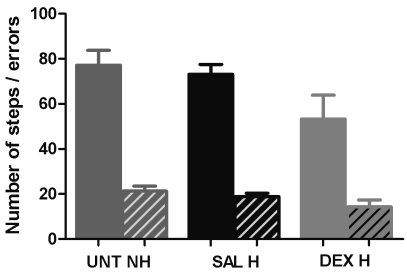

Fig 10. Total number of steps and errors in foot fault test. DEX H animals tend to be overall less active compared to SAL H and UNT NH animals, however error rate is comparable between groups.

Water maze performance

Repeated measures ANOVA revealed a significant main effect of time ($F(3,72) = 14.69$, $p < .001$) and handling ($F(1,24) = 7.55$, $p = .011$) indicating that UNT NH animals show higher latency to reach the platform compared to H animals during spatial learning (day 1-4). Performance during reversal learning on day 5 was not different between groups (fig. 11A). Additionally, on day 1 of spatial learning 65% of UNT NH animals did not reach the platform within 120 seconds and needed to be guided there, compared to only 25% of SAL H and 33% of DEX H animals (fig. 11B). Chi-Square analysis revealed that H animals need significantly less guiding on day 1 compared to UNT NH (χ^2 (1) 6.857, $p = .009$).

Body Weight

Analysis of body weight of SAL H and DEX H animals shows a significant effect of time ($F(15,240) = 118.89$, $p < .001$) and a time x drug treatment interaction ($F(15,240) = 2.08$, $p = .011$). Post-hoc analysis per time point revealed that the weight of DEX-treated animals is significantly less up to week 44 (all $p < .05$). When age increases further, differences between SAL H and DEX H animals disappear.

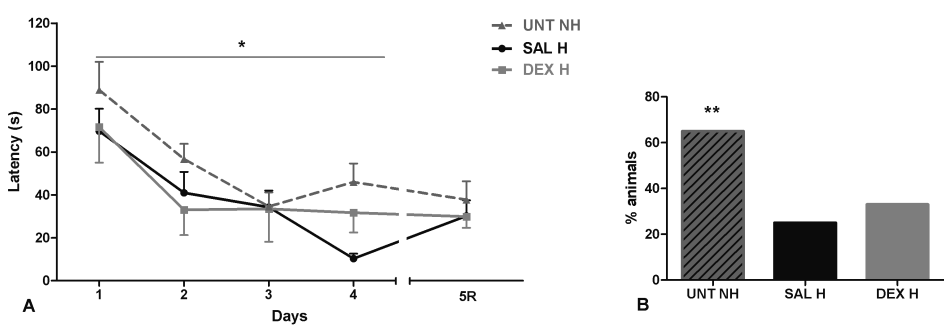

Fig 11. Spatial learning in the Morris Water Maze. Latency to reach the platform (A): performance per day (as average of 2 trials/day) on day 1-4 (spatial learning) and day 5 (reversal learning). H animals reach the platform significantly faster compared to NH animals on days 1-4. Performance during reversal learning on day 5 is not different. On day 1 a significantly higher percentage of UNT NH compared to H animals need to be guided to the platform (B). * $p < .05$ ** $p < .01$

When UNT NH animals are included in the analysis, repeated measures ANOVA shows a main effect of time ($F(2.96,71.04) = 199.62$, $p < .001$), without effects of drug treatment or handling (fig. 12).

Survival

Although DEX H animals appear to die at a younger age and reach 50% survival several months before SAL H and UNT NH animals, Kaplan Meier analysis does not indicate significant differences in survival between treatment groups up to the age of 26 months (fig.13).

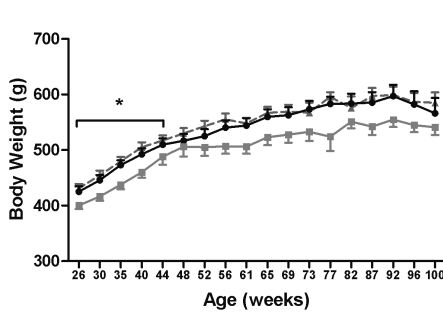

Fig 12. Body weights of SAL H, DEX H and UNT NH animals from week 26 – 100. Up to 44 weeks of age, DEX H animals show lower body weight compared to SAL H. However with increasing age differences in body weight disappear. * $p < .05$ (SAL H vs DEX H)

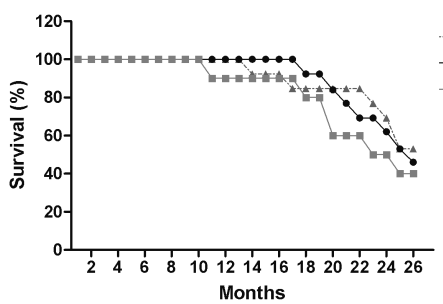

Fig 13. Survival curves of UNT NH, SAL H and DEX H animals up to 26 months of age. Although DEX H animals appear to have the steepest survival curve, differences between groups do not reach statistical significance.

DISCUSSION

The goal of this study was to investigate the impact of neonatal glucocorticoid treatment on development, as well as on adult and aged phenotype in rats. We reported that neonatal DEX treatment resulted in developmental alterations in body weight, eye opening and fur development. However, we did not observe the frequently described alterations in cognitive performance and stress responsiveness in adulthood. Interestingly, the current experimental design consisted, besides manipulations necessary for injections on pnd 1, 2 and 3, of a substantial amount of neonatal handling during the full postnatal period (pnd 1-21) because of daily weighing and marking for discrimination between

individual pups receiving different treatments within the litter. Therefore, we included an untreated non-handled control group - originating from the same cohort of animals - in part II of the experiment to investigate the impact of neonatal handling. Indeed we observed on several parameters (endocrine and behavioural) throughout the lifespan a profound effect of neonatal handling without substantial differences between SAL and DEX-treated animals. Therefore we suggested that the impact of neonatal handling may have interacted with and potentially protected against DEX-induced alterations.

Neonatal glucocorticoid treatment: impact on development

The impact of neonatal glucocorticoid treatment on development has been frequently reported. Reduced growth, similar to the findings in the current study, has been demonstrated in human as well as animal studies (21, 23-26). Although we have not investigated the underlying mechanism, others have suggested that DEX prevents adequate growth by inducing protein catabolism during a developmental period normally characterized by low levels of circulating glucocorticoids, thus promoting an anabolic state (27).

Additionally, Vazquez and colleagues suggested that the reduction in weight gain could be attributed to an inability of DEX pups to get milk from the mother due to poor suckling. Their findings also demonstrate that DEX treated rat pups have lower post-weaning food intake (28). Human studies however demonstrate that the reduced growth seen in infants receiving DEX treatment cannot be explained by decreased energy intake or increased expenditure, but may be due to differences in the composition of newly accreted tissue due to a shift in intermediate metabolism (29).

Besides reduced growth, other developmental alterations are associated with neonatal glucocorticoid treatment. We report changes in eye opening and fur development which have also been reported in previous literature (23). The mechanism underlying these findings is still poorly understood. Neurodevelopmental delay following DEX treatment is frequently explained as being the result of inhibition of normal myelination processes (23). Peripherally, the impact of DEX has been often studied in the context of lung development. During normal development, glucocorticoids regulate the degree of proliferation and differentiation. Glucocorticoid-insufficient (CRH knock-out) animals, suffering perinatally from abnormal pulmonary development due to hyper-proliferation, can be rescued by exogenous glucocorticoid treatment (30). In premature infants, glucocorticoid treatment can enhance lung maturation (31) by stimulating differentiation of epithelial cells (32). Exogenous glucocorticoid administration during normal development leads to hypo-proliferation, as well as to pulmonary epithelial maturation (33).

All together, it appears that glucocorticoid exposure enhances maturation and differentiation at the expense of growth and proliferation, as is reviewed by Bolt (34), which is either beneficial or detrimental depending on the developmental context. Similar alterations in other tissues might underlie the developmental alterations observed in the current study.

Long-lasting effects on adult phenotype

Many studies have demonstrated long-lasting effects of neonatal DEX treatment on adult phenotype in terms of cognitive performance, hippocampal function, emotionality and stress responsiveness (19, 22, 28). Interestingly we did not observe such effects in the current study. Does this mean that DEX-induced alterations are not as adverse as suggested previously? There might be other factors playing a role in determining phenotypic outcome. Many of the previously mentioned studies have used Sprague-Dawley or Wistar rats as subjects, which might have a different sensitivity for neonatal glucocorticoid exposure, or early-life experiences in general, compared to Long-Evans rats. A comparable phenomenon has been reported in mice, in terms of sensitivity to the programming effects of maternal care on drug self-administration and depression-like behaviour (35). Additionally, besides the use of different strains, there is substantial variation in the treatment regimen among studies investigating the impact of neonatal exposure to glucocorticoids. Dosing and time of treatment varies greatly, as well as post weaning housing conditions. These factors are likely to contribute to determining treatment outcome.

Furthermore, the DEX-induced effects reported previously might be specific for certain behavioural paradigms. In contrast to many studies testing spatial learning using a water maze paradigm (19, 36), we initially used a circular hole board. It is known that the water maze, compared to the hole board results in substantially higher HPA axis activation (37). Altered expression of the glucocorticoid receptor (GR) after neonatal DEX treatment (38), might explain changes in water maze performance between SAL and DEX treated animals without differences on the hole board.

Surprisingly, we observed slightly enhanced performance on certain aspects of circular hole board learning in DEX compared to SAL-treated animals. Moreover, when tested at 22 months of age in the water maze, DEX-treated animals overall do not show learning impairments, although SAL animals appear to continue to improve performance on the last learning day (day 4) in contrast to DEX animals who reach their maximum performance on day 2 without further improvement.

Additionally, the type of stressor appears to determine the DEX-induced effect on the endocrine response. Although DEX-treated animals have been reported to show a blunted CORT response to novelty stress; CORT levels did not differ from SAL-treated animals after experiencing conditioned fear (20). Immobilization stress (used in this study) can be considered a severe stressor; leading to a greater HPA axis activation compared to other stressors (39). This potentially reduced the likelihood to reveal differences between SAL and DEX-treated animals in stress responsiveness, due to a ceiling effect.

However, Felszeghy and colleagues did report a suppressed elevation of both ACTH and CORT in response to restraint stress in adult rats that were neonatally exposed to DEX, but on pnd 1, 3 and 5 (40). Contrarily, other studies have reported a prolonged (rather than blunted) CORT response in DEX compared to SAL-treated animals after crowding stress (23), indicating that the DEX-induced

effect on the endocrine stress response is highly dependent on the type of stressor applied.

Hence, another factor potentially contributing to the somewhat surprising differences in outcome lies in the experimental design. As mentioned before, our design involved a substantial amount of neonatal handling. Besides manipulations necessary for injections on pnd 1, 2 and 3, SAL and DEX treated animals were daily weighed and marked during the full postnatal period in order to discriminate between animals receiving different treatments within the litter. Other studies investigating the long-term effects of neonatal DEX-treatment have used either a between-litter design (21) or a within-litter design with use of another type of marking (41) reducing the amount of daily handling.

The within-litter design was chosen because it has the advantage of having both the genetic contribution as well as the shared maternal environment from a given litter represented in both treatment groups. Daily marking using a non-permanent odourless marker was chosen since it is less invasive compared to a tattoo or toe clip. However, marking did lead to a substantial amount of daily handling of the neonate up to weaning age.

Impact of neonatal handling

More than 6 decades ago, it was discovered that brief (3-15 min) daily separations between rodent mother and pup between pnd 1 and 21 had long-lasting impact on adult stress phenotype (42). Follow-up studies demonstrated that these manipulations resulted in HPA axis hypo-responsiveness (10, 43, 44) likely mediated by altered GR expression (45). Additionally, reduced emotionality (43), and increased cognitive performance (46) were reported in handled (H) rats compared to rats raised in undisturbed laboratory conditions, i.e. non-handled (NH).

The data suggest that the effects of H might have potentially compensated for certain DEX-induced alterations. Regarding cognitive performance, H has been shown to improve spatial learning on the circular hole board (47) and in the water maze, an effect that lasts up to old age (48, 49) like we observed in the current study. These findings suggest that the effects of H might have overruled DEX-induced adverse effects on spatial learning.

Interestingly, the effects of H and DEX treatment (20, 22, 44) on endocrine stress responsiveness are suggested to point in the same direction, i.e. they both result in a blunted response and enhanced feedback sensitivity (although likely via different mechanisms). In the current experiment, we observed the frequently described effect of H on the stress response without additional effect of DEX exposure. The effect of H might have overruled a potential HPA suppressing effect of DEX. If both of these individual effects are present, they apparently do not work synergistically, since DEX H animals do not differ from SAL H in their endocrine responsiveness.

Neonatal glucocorticoid treatment has resulted in inconsistent findings regarding PPI phenotype. Ferguson and colleagues report no effect of DEX treatment (50) whereas Hauser and colleagues report an increase in PPI after

prenatal DEX treatment, which was not replicated (51). Additionally, our data indicate no differences between SAL and DEX treated animals. In contrast to other studies demonstrating no effect of H on PPI (52), we report that H enhanced PPI substantially.

Interestingly, other pre-weaning and post-weaning manipulations like maternal deprivation and social isolation (53, 54) have been reported to affect PPI. In this study - besides differences in postnatal experience - there is also a substantial difference in post-weaning manipulation between the H and UNT NH animals up to the age of 6 months (due to extensive behavioural testing of the H animals), which could explain differences in PPI between these groups. However, a study from our laboratory investigating this effect (post-weaning manipulation due to exposure to behavioural testing) indicates no differences between animals with a comparable postnatal experience which are either extensively tested or undisturbed during the post-weaning period (Claessens et al, unpublished data) suggesting that the differences in PPI in the current study can be attributed to early-life experiences.

We did not report DEX induced alterations in motor performance in middle aged animals, in contrast to several human and animal studies showing DEX induced effects on neuro-motor development and performance (55, 56). Findings from human studies have suggested that these impairments cannot be fully attributed to DEX treatment, but that medical and socio-demographic factors other than GC treatment also contribute to the phenotype (57). Although others have suggested that motor performance in aged animals is not affected by H (49), we demonstrate beneficial effects of H on motor learning in middle aged rats, potentially interacting with or compensating for the effects of neonatal DEX exposure.

Finally, we report that DEX treated animals appear to have a steeper survival curve, but in contrast with other rodent studies showing shortening of the lifespan after perinatal DEX treatment (21, 58, 59), this effect did not reach statistical significance. Whether a DEX effect might have interacted with potential beneficial effects of H remains to be investigated. However, we did not demonstrate an overall effect of H on lifespan.

The effects of H are suggested to be mediated via enhancing maternal care (60). It has to be noted that the postnatal manipulations in the current study are not identical to H as it is known in literature. Besides daily periods of brief separation, the offspring was exposed to the procedure of marking and weighing. Altogether, this can be considered a more abundant form of handling, which has been shown to interact with other developmental experiences (61). Unfortunately we have not studied maternal behaviour in the current experiment, but we did observe increased maternal licking and grooming directed at H compared to NH offspring under comparable experimental conditions (Claessens et al, unpublished data). Interestingly, Brabham and colleagues reported that certain (prenatal) DEX induced effects can be normalized by enhanced levels of maternal care during the postnatal period (36). In line with these findings we suggest that enhanced maternal care, as a result of H, is likely to contribute to 'rescuing' the DEX phenotype.

CONCLUDING REMARKS

We did not observe the frequently reported programming effects of DEX treatment on adult phenotype, while DEX clearly affected the developmental trajectory and body weight. However, we did observe profound and long-lasting effects of H in both SAL and DEX treated animals. Since, to our knowledge, this treatment design (combining H and glucocorticoid treatment) has not been used before, we suggest that H might serve as a protective intervention, potentially compensating for the impact of neonatal DEX exposure. From these findings we cannot conclude that the effects of H have overruled DEX-induced alterations or that the current DEX-treatment would have resulted in a different outcome if administered in a NH context. Whether H can in fact compensate for DEX-induced alterations in adult phenotype remains to be investigated in follow-up studies using appropriate control groups investigating the impact of DEX in a handing and non-handling context.

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CHAPTER 5

Early handling modulates outcome of neonatal glucocorticoid exposure

Sanne E.F. Claessens, Nikolaos P. Daskalakis,
Melly S. Oitzl and E. Ronald de Kloet

Division of Medical Pharmacology, LACDR/LUMC, Leiden University, The Netherlands

Submitted

ABSTRACT

Synthetic glucocorticoids are frequently used to prevent respiratory disorders in prematurely born infants. Besides the short-term benefit on lung function, numerous human and animal follow-up studies have reported adverse neurodevelopmental side effects. In contrast to these reports we recently showed a relatively mild outcome after neonatal dexamethasone treatment using a rat model. The aim of the current study was to investigate whether neonatal handling, which was an inevitable component of our experimental design, might serve as an intervention strategy modulating the adverse effects of dexamethasone treatment.

Rat pups were injected with dexamethasone or saline on postnatal days 1, 2 and 3, and additionally daily handled or left undisturbed until postnatal day 21. Maternal care was observed during the first week of life and was enhanced in response to handling. Eye opening was accelerated and body weight reduced in dexamethasone treated animals. In adulthood, we report that although dexamethasone treatment and handling yielded comparable effects on stress-induced CORT response and startle reactivity, acquisition of fear was only affected by handling. Dexamethasone treatment reduced the sensitivity for beneficial effects of handling on pre-pulse inhibition. Non-handled animals appeared more susceptible to the impact of dexamethasone treatment compared to handled animals, as was demonstrated for spatial learning in the water maze. Moreover, dexamethasone treatment only impaired spatial orientation in the T-maze in non-handled animals.

These findings emphasize that the outcome of neonatal glucocorticoid exposure is not deterministic and strongly interacts with other components of the postnatal environment.

INTRODUCTION

Synthetic glucocorticoids such as dexamethasone (DEX) are frequently used to enhance lung function in prematurely born infants. Although some studies indeed showed beneficial effects of glucocorticoid treatment (1) leading to a decreased incidence and severity of bronchopulmonary dysplasia, others however failed to do so or showed only modest effects (2). Moreover, follow-up studies of prematurely born infants treated with glucocorticoids have shown pervasive adverse neurodevelopmental effects (3, 4). Randomized placebo-controlled trials reported that glucocorticoid treatment led to an increased incidence of neurodevelopmental impairment (3), and resulted in poor motor skills as well as lower IQ scores compared to untreated controls (5). Therefore there has been growing concern whether the short-term benefits of glucocorticoid treatment outweigh the adverse side effects leading to neurodevelopmental impairment (6).

In line with evidence from human studies, rodent studies demonstrated that perinatal glucocorticoid treatment resulted in long-lasting alterations in cognitive performance and hippocampal function (7-10), stress responsiveness (11, 12), social behaviour (13) and to a significantly shortened lifespan (10, 14). Previous rodent studies in our laboratory did show developmental alterations after neonatal DEX treatment in terms of brain development, body weight and eye opening. However, the long term consequences for behavioural phenotype were relatively mild and in some cases even beneficial (chapter 4 of this thesis).

Interestingly, our experimental design consisted, besides manipulations necessary for drug treatment, of a substantial amount of neonatal handling during the full postnatal period. This was the result of daily weighing and marking for discrimination between individual pups receiving different treatments according to a within-litter design. Other studies investigating the long-term effects of neonatal DEX-treatment have used either a between-litter design (14) or a within-litter design with use of another type of marking (15) reducing the amount of daily handling.

It is well known that daily handling during the postnatal period attenuates stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis (16-19), reduces emotionality (18), and enhances cognitive performance (20). Moreover, we have previously reported the long-lasting impact of neonatal handling - inherent to the dexamethasone-treatment design - throughout the lifespan, suggesting an interaction with the effects of DEX treatment (chapter 4 of this thesis). Therefore, it is hypothesized that the effects of handling might have potentially compensated for certain DEX-induced alterations. To investigate whether handling of the neonate can indeed reverse neonatal DEX-induced alterations in adult phenotype we have studied the effects of glucocorticoid treatment in a handling vs a non handling context.

MATERIAL AND METHODS

Animals

Adult female and male Long Evans rats from our breeding population (originally obtained from Janvier, France) were used as breeders. Two females were mated with one male for 10 days in type IV polycarbonate cages (59x38x20cm) containing sawdust bedding and tissues. Food (RM3, Special Diet Services, Witham, Essex, UK) and water (8 ml 25% HCl /10 L tap water) were provided *ad libitum*. Animals were maintained on a 11-h light : 13-h dark cycle with lights on at 08.30h, in a temperature (21± 2°C) and humidity (55 ± 5%) controlled room. After breeding, pregnant females were individually housed. Females were checked daily for presence of pups. If pups were present, the day of birth for that particular litter was defined as postnatal day 0 (pnd 0). On pnd 1, litters were culled to 8 pups (4 male and 4 female) and randomly assigned to the Handling (H) or Non Handling (NH) group. Cages were cleaned once on pnd 10 and after weaning once weekly. After weaning (pnd 22) animals were group housed with same sex littermates. Animal experiments were approved by the Local Committee for Animal Health, Ethics and Research of Leiden University and carried out in accordance with European Communities Council Directive (86/609/EEC).

Drug Treatment

Male pups were randomly assigned to either the saline (SAL) or the dexamethasone (DEX) group using a within litter design. Pups in the DEX group were subcutaneously (SC) injected with dexamethasone-21-phosphate (Sigma Aldrich, Zwijndrecht, The Netherlands) on pnd 1 (0,5 µg/g body weight), pnd 2 (0,3 µg/g) and pnd 3 (0,1 µg/g). Pups in the SAL groups were injected with equivalent volumes of sterile and pyrogen free saline (SAL). In order to prevent bias for the dam to show enhanced attention for injected or non-injected offspring, all female littermates were injected with SAL on pnd 1, 2 and 3. Pups were marked for identification with a toe clip on pnd 1. Drug treatment always took place between 9:00 and 11:00.

Handling

From pnd 1-21 H pups underwent daily 15 min separations from the dam. At the onset of each separation, pups were removed altogether from the home cage, and placed in a type IIL polycarbonate cage (36,5x20,5x14cm) that was brought to an adjacent room, where it was placed on a heating pad. Following 15 min of separation, all pups were returned to the housing room and reunited with the dam in the home cage. Handling always took place between 12:00 and 13:00. NH pups were left undisturbed from pnd 1-21 except for a cage change on pnd 10.

Maternal Care

The maternal behaviour of each dam was observed and scored for five 60 minute periods per day during the first 7 days postpartum using a procedure as described by Champagne *et al* (2003) (21). Observations were performed at three periods

during the light phase (13:00, 15.30, 18:00) and two periods during the dark phase (07:00 and 20:00) of the light cycle. Within each observation period the behaviour of each mother was scored every 3 minutes (20 observations per period, 100 observations per day, 700 observations for the first 7 days postpartum). The following maternal behaviours were scored: a) Licking and grooming (LG) of the pups, b) Arched back nursing/blanket nursing/passive nursing, c) Mother away from nest/no maternal contact. The data were analyzed as the percentage of observations in which dams displayed one of the behaviours described above.

Postnatal development

Postnatal development (body weight and eye opening) was monitored in H pups during H procedure. For eye opening a scale from 0-2 was used indicating: 0 = closed, 1 = partial opening, 2 = fully open (adapted from Flagel, Vazquez, 2001).

Adult Phenotype

Animals were tested according to the schedule depicted in figure 1.

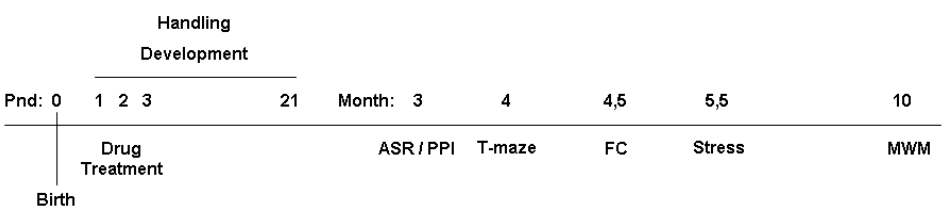

Fig 1. Timeline experiments. Pnd: postnatal day; ASR: acoustic startle reactivity; PPI: prepulse inhibition; FC: fear conditioning; Stress: endocrine response to restraint stress; MWM: morris water maze.

Acoustic Startle Reactivity and Prepulse Inhibition

Prepulse inhibition was measured at 3 months of age. Animals were brought to the testing room and allowed to habituate for 45 min. After habituation they were placed in a startle recording apparatus (SR-LAB, San Diego Instruments, CA, USA) containing a transparent Plexiglas tube (diameter 8.7 cm, length 20.5 cm) mounted on a Plexiglas base. Sounds were presented by a speaker and movement of the animals was detected by a piezoelectric accelerometer mounted below the Plexiglas tube and recorded by a computer. Testing started with a 5 min habituation session with background white noise of 70 dB[A]. Animals were first presented with six pulse alone trials (117 dB[A]) followed by 39 trials comprising different trial types according to a pseudo-randomized schedule with an inter-trial interval of 10-20 sec. Trial types: background white noise alone, prepulse alone using intensities of 2, 4, 8, 16 dB[A] above background noise (i.e. 72, 74, 78 and 86 dB[A]), pulse alone (117 dB[A]) or a combination of one of the four prepulses plus pulse. Finally, animals were again exposed to five pulse alone trials. The

duration of the prepulses was 20 ms, duration of the pulses was 40 ms. Prepulse to pulse interval was 100 ms. Startle activity was measured during 100 ms after onset of the pulse. The percentage of PPI at the different prepulse intensities was calculated as [100-(100 x startle amplitude at prepulse trial)/ (startle amplitude at startle pulse-alone trial)]. Speakers were calibrated every day, and boxes were cleaned with a 10% ethanol solution after every session to eliminate odour cues. Experiments were performed between 09:00 and 13:00 to minimize circadian influence.

T-maze

Spatial orientation in the T-maze was investigated at 4 months of age. The T-maze consisted of three unequally sized arms made of Plexiglas. The length of the start arm was 75 cm, whereas the other arms were both 32 cm in length. The width and height of the arms were 12 and 20 cm respectively. During training, rats were placed in the start arm facing the outer wall and were allowed to explore the start arm and one of the two other arms for 10 minutes. Half of the animals were allowed to visit the left and the other half the right arm to reduce the influence of a preference of the rats for one side. After a delay of 2.5 hours, rats were placed back in the T-maze and were allowed to explore all three arms for 5 minutes. The duration and frequency of visiting the familiar or new arm during re-exposure were measured using EthoVision XT (Noldus Information Technology, Wageningen, The Netherlands). T-maze experiments were performed between 09:00 and 13:30 to minimize circadian influence. The experimental apparatus was cleaned with a 10% ethanol solution between all sessions to eliminate odour cues. To reduce the effect of stress due to testing order within the cage, one animal per litter was tested per day.

Contextual Fear Conditioning

Contextual fear memory was assessed at 4,5 months of age. The shock box (40x40x50 cm) consisted of black Plexiglas walls and a stainless steel rod floor, connected to a shock generator. On day 1 the animal was transferred from the housing room to the adjacent test room where it was placed in the shock box. After a 2 min delay it was exposed to a foot shock of 0.6 mA (duration 2 sec). After the shock the animal remained in the shock box for 2 minutes, after which it was removed from the box and transferred back to the home cage.

24 hours later (day 2), the animal was re-exposed to the shock box for 4 min, however without receiving a foot shock. An overhead video camera recorded behaviour of the animals throughout all sessions. Behaviour was analyzed by an observer unaware of treatment conditions using The Observer 9.0 XT (Noldus Information Technology, Wageningen, The Netherlands). The following behaviours were scored (1) freezing (lack of all body movement except those necessary for breathing), (2) scanning (lack of movements, except lateral head movements and movements necessary for breathing), (3) rearing, (4) walking, and (5) sitting. Behaviour was analyzed during 3 distinct time periods: (1) 2 min before

shock, (2) 2 min after shock, (3) 4 min re-exposure. All testing took place between 10:00 and 13:00 to minimize circadian influence. The shock box was cleaned with a 10% ethanol solution between all sessions to eliminate odour cues. To reduce the effect of stress due to testing order within the cage, one animal per litter was tested per day.

Endocrine response to restraint stress

At 5,5 months of age, endocrine response to an acute restraint stressor was tested. One day prior to restraint stress a basal blood sample was taken from the tail vein. The next day animals were exposed to 10 min restraint stress by placing them in a custom made restrainer restricting body movement. Blood samples were taken at 2, 5, 10, 30, 60, and 120 min.

Hormone Analysis

Blood samples were collected in EDTA coated tubes (Microvette CB 300 K2E, Sarstedt, Germany). Samples were kept on ice and centrifuged for 15 min at 13000 rpm at 4°C. Plasma was transferred to Eppendorf tubes and stored at -20°C until further analysis. Plasma and corticosterone (CORT) concentration was measured using a commercially available radio immuno assay (RIA) kit containing ^{125}Iodine labelled CORT (MP Biomedicals Inc., USA). All samples were processed in the same assay to exclude inter-assay variability.

Water maze performance

At 10 months of age spatial learning in a water maze was tested. Animals were put, without prior training in a pool (150 cm diameter) filled with 30 cm of water (21°C) made opaque by adding 3 spoons of latex paint. A platform (10 cm diameter) was hidden 1 cm below the surface of the water and was positioned in the NE quadrant. Animals were given 3 daily trials (inter-trial interval 15 min) to find the platform on day 1, 2 and 3. On day 4, two more training trials were given, followed by a probe trial without platform present. On day 5, animals were given 3 reversal trials with the platform located in the position opposite (SW quadrant) from where it was during training. Trials always started in one of the 3 quadrants where the platform was not located in a pseudo-randomized fashion, following the same order of start position for every animal. If an animal did not reach the platform within 2 minutes, it was gently guided there by the experimenter. Animals were allowed to stay on the platform for 15 sec after finding it or being guided there. Swim patterns were tracked by an overhead camera and later analysed using EthoVision XT (Noldus Information Technology, Wageningen, The Netherlands). Latency and distance to platform as well as latency, frequency and duration in target and other quadrants, or in close proximity to the platform (or the location where the platform in previous trials) were analyzed during training and reversal trials, as well as swim patterns during probe trials. After every swim trial, animals were dried with a towel and kept in a clean cage lined with clean dry tissue paper

that was placed on a heating pad. All trials took place between 10:00 and 13:00 to minimize circadian influence.

Data analysis

Statistical analysis was performed using SPSS 17.0. Data are presented as mean +/- SEM. Data were analysed using two-way repeated measures ANOVA with time (age, trial, day or min) as within and drug treatment and handling as between subject factors, one-way ANOVA to compare H to NH animals (in case of maternal LG data), or two-way ANOVA to measure a main effect of drug treatment (SAL, DEX) and handling (NH, H), or an interaction between drug treatment and handling. If an interaction was observed, post-hoc pair-wise comparisons were performed. For the T-maze experiments, differences within each group were tested using a paired t-test. Level of significance was set at $p < .05$. Where needed corrections for multiple testing and violation of sphericity were conducted. Graphs were made using GraphPad Prism®.

RESULTS

Maternal care

H pups received significantly higher levels of maternal LG compared to NH pups directly following exposure to the handling procedure ($F(1,39) = 12.72$, $p = .001$, fig 2A). However, overall level of LG did not differ significantly between NH and H litters (fig 2B).

Body weight and postnatal development

DEX treated animals weighted significantly less throughout the postnatal period ($F(1,20) = 90.25$, $p < .001$, fig 3A). For obvious reasons this was monitored only in H animals. During the post-weaning period (fig 3B), NH animals tended to

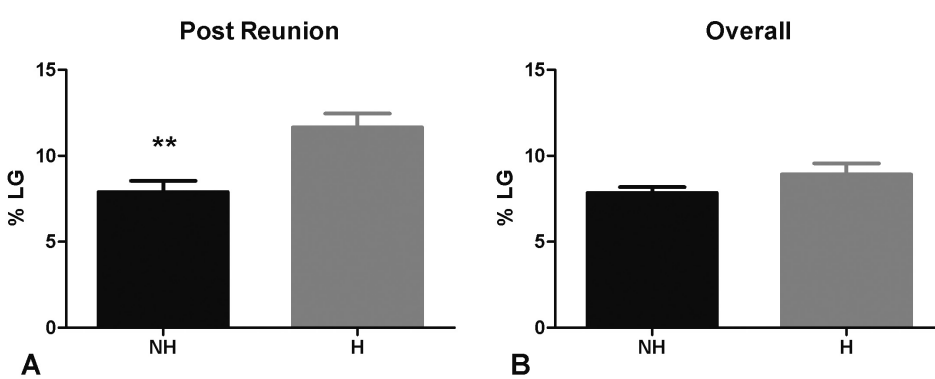

Fig 2. Maternal licking and grooming (LG) directly following the handling procedure (A) and overall (B). Displayed are mean +/- SEM. ** $p < .01$

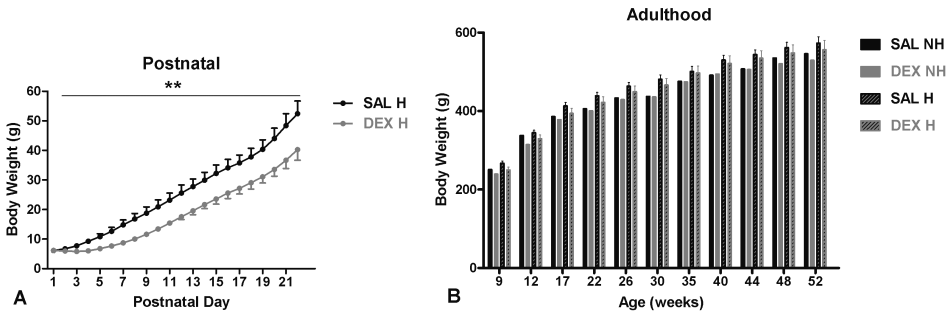

Fig 3. DEX treatment significantly reduced body weight throughout the postnatal period (A). In adulthood, no effect of DEX treatment was observed. H however tended (ns) to result in higher body weight (B). Data represent mean ± SEM. ** p < .01

have a lower body weight than H animals, although this effect was not statistically significant (F(1,32) = 4.12, p = .051).

For eye opening we observed a significant main effect of drug treatment (F(1,19) = 40.66, p < .001, fig 4) indicating that DEX treated animals show an acceleration in eye opening. For obvious reasons this was monitored only in H animals. We also observed a significant time x drug treatment interaction (F(20,380) = 17.29, p < .001). Post-hoc analysis per time point revealed that DEX treated animals showed enhanced levels of eye opening compared to SAL treated animals on pnd 8 (p = .019), 9, 10, 11, 12 (p < .01).

Adult phenotype

Acoustic Startle Reactivity

Acoustic startle reactivity (ASR) to the first five pulses revealed a main effect of handling (F(1,37) = 9.047, p = .005) and a drug treatment x handling interaction (F(1,37) = 13,497, p < .001; fig 5A, black bars). Post-hoc pair-wise comparisons revealed higher ASR in SAL NH animals compared to SAL H (p = .011) and DEX NH (p = .023), indicating that both H and DEX treatment reduced initial startle reactivity. No main effect of handling, drug treatment or an interaction effect was

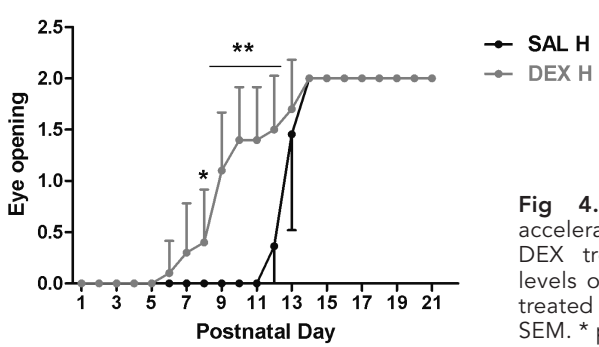

Fig 4. DEX treatment resulted in accelerated eye opening, on pnd 8-12 DEX treated animals show enhanced levels of eye opening compared to SAL treated animals. Data represent mean ± SEM. * p < .05, ** p < .01

observed for the last five pulses where all groups showed similar startle reactivity (fig 5A, white bars).

Habituation of ASR was calculated based on the difference in ASR to the first and the last five pulses. Main effects for handling ($F(1,33) = 8.478$, $p = .006$) and drug treatment ($F(1,33) = 10.901$, $p = .002$) were observed, indicating that both H and DEX treatment reduced habituation of the acoustic startle response (fig 5B), an effect that appears to be mostly driven by the high initial ASR in SAL NH animals.

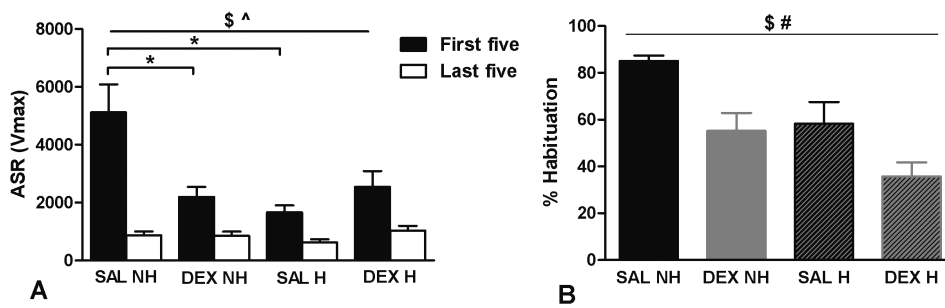

Fig 5. Acoustic startle reactivity (ASR) to the first five (black bars) and last five (white bars) pulses (A) and habituation of ASR (B). Data represent mean ± SEM. $ main effect of handling $p < .01$, # main effect of drug treatment $p < .01$, ^ drug treatment x handling interaction $p < .01$. Results of post-hoc pair-wise comparisons: * $p < 0.05$.

Prepulse Inhibition (PPI)

Analysis of PPI data showed a significant drug treatment x handling interaction ($F(1,35) = 4.81$, $p = .035$, fig 6) for PP2 (prepulse 2 dB[A] above background). Post-hoc pair-wise comparisons revealed significantly lower PPI in SAL NH animals as compared to SAL H animals ($p = .021$) indicating that H enhanced PPI in SAL treated but did not affect DEX treated animals. We did not observe main effects of drug treatment or handling on PPI. Effects were comparable for PP4 (4 dB[A] above background), however post-hoc testing showed a trend towards significance. No effects of DEX treatment or H were observed for PP8 and PP16.

Spatial orientation in the T-maze

Since exploratory behaviour was reduced after the first minute of re-exposure to the T-maze, we focused on behaviour during the first minute. All animals, except DEX NH, showed the expected preference for the new versus the familiar arm of the T-maze, indicating that this DEX-induced effect on hippocampal performance can be reversed by handling. Paired T-tests showed that all groups except for DEX NH spent significantly more time in the new compared to the familiar arm (SAL NH: $p = .028$, SAL H: $p = .002$, DEX H: $p < .001$, fig 7).

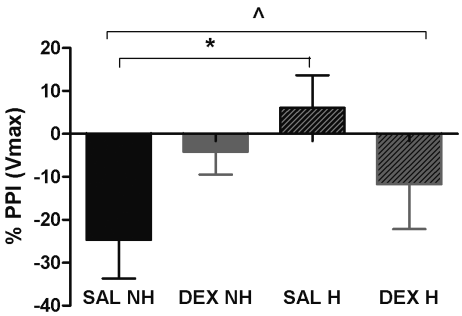

Fig 6. Prepulse inhibition for PP2 (2 dB[A] above background). Handling significantly enhanced PPI in SAL treated animals without affecting DEX treated animals. Data represent mean ± SEM. ^ drug treatment x handling interaction $p < .05$. Results of post-hoc pair-wise comparisons: * $p < .05$.

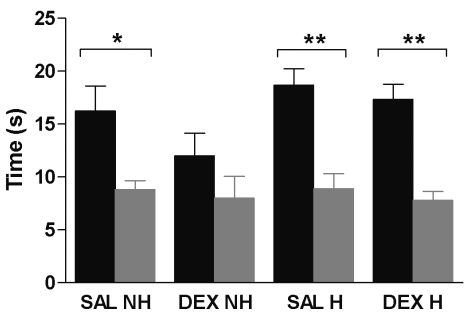

Fig 7. Spatial orientation in the T-Maze. All groups except DEX NH spent significantly more time in the new compared to the familiar arm during re-exposure to T-maze. Data represent mean ± SEM. * $p < .05$, ** $p < .01$.

Contextual Fear Conditioning

No significant differences in behaviour were observed between groups prior to shock administration**.** Directly after shock exposure, a main effect of handling was observed for freezing ($F(1,36) = 14{,}731$, $p < .001$, fig 8A) indicating that H animals showed less freezing compared to NH animals. During re-exposure (24h later), a main effect of handling was observed for freezing ($F(1,36) = 8.892$, $p = .005$, fig 8B) indicating that again, H animals showed less freezing compared to NH animals. Three animals did not receive a shock and were therefore excluded from the analysis.

Endocrine stress responsiveness

Repeated measures ANOVA revealed a main effect of time ($F(2.39, 69.20) = 184.73$, $p < .001$) and a time x drug treatment x handling interaction ($F(2.39,69.20) = 4.56$, $p = .010$). Post-hoc analysis per time point indicated at t = 120 min a significant drug treatment x handling interaction ($F(3,42) = 3.55$, $p = .023$). At t = 120 SAL NH animals showed significantly higher CORT levels compared to DEX NH ($p = .005$) and SAL H ($p = .009$). The difference compared to DEX H was not statistically significant ($p = .071$). This indicates that both handling and DEX treatment resulted in enhanced negative feedback of the HPA axis in response to acute restraint stress (fig 9).

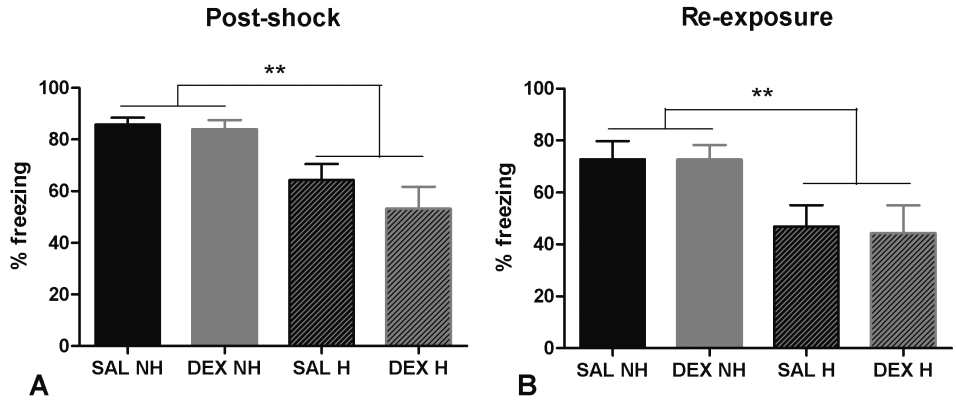

Fig 8. Freezing behaviour directly following shock exposure (A) and during re-exposure to shock context (B). Data represent mean ± SEM. Handled animals display significantly lower levels of freezing both directly after shock exposure and during re-exposure to the shock context. **: main effect of handling, $p < .01$

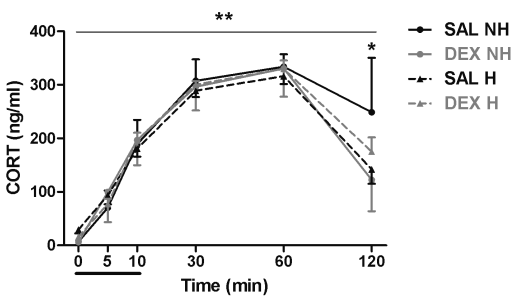

Fig 9. Corticosterone (CORT) levels before, during and following exposure to 10 min restraint stress (indicated with horizontal black bar). Both handling and DEX treatment result in enhanced negative feedback of the HPA axis at t = 120 min. Data represent mean ± SEM. ** time x drug treatment x handling interaction $p = .01$, * drug treatment x handling interaction at t = 120, $p < .05$

Spatial learning in the Morris Water Maze

Repeated measures ANOVA of spatial learning revealed a main effect of time ($F(2.09,77.35) = 127.62$, $p < .001$) and a main effect of drug treatment ($F(1,37) = 5.41$, $p = .026$), indicating that SAL treated animals show overall a longer latency to find the platform compared to DEX treated animals during spatial learning on days 1 to 4. There was a trend towards a drug treatment x handling interaction ($F(1,37) = 4.07$, $p = .051$). Post-hoc analysis showed that overall SAL NH animals need more time to find the platform compared to DEX NH ($p = .026$), an effect that is mostly driven by performance on day 1 (fig 10A). When day 1 is analysed into more detail (per trial, see fig 10B) we observed a main effect of time ($F(2,74) = 26.71$, $p < .001$), and drug treatment ($F(1,37) = 8.74$, $p = .005$) and a drug treatment x handling interaction ($F(1,37) = 6.80$, $p = .013$). Post-hoc analysis shows that SAL NH animals need significantly more time to find the platform compared to DEX NH animals ($p = .003$) whereas the other groups do not differ significantly. This indicates that DEX treatment affects mostly NH animals. However, although

DEX NH animals are significantly faster compared to SAL NH, the slope of the learning curves is highly comparable.

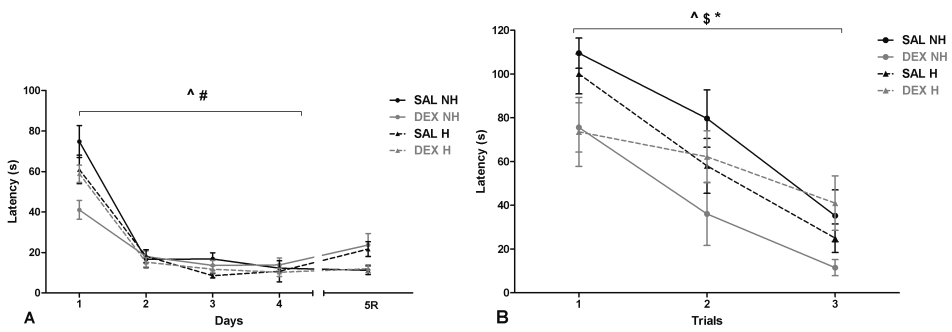

Fig 10. Spatial learning in the water maze. Overall, DEX treated animals had a shorter latency to find the platform compared to SAL treated animals. This effect was mostly driven by a difference between SAL NH and DEX NH on training day 1 (A). When day 1 was analysed per trial we observed that DEX treatment affected NH animals, without affecting H animals. DEX NH animals are faster on all trials, including trial 1 (B). ^ main effect of time $p < .01$, # main effect of drug treatment $p < .05$, $ main effect of drug treatment $p < .01$ * drug treatment x handling interaction $p < .05$.

DISCUSSION

The aim of this study was to investigate the impact of neonatal glucocorticoid treatment in an H vs NH context, in order to answer the question if H compensates for the effects of neonatal DEX treatment. We observed DEX-induced developmental alterations, in terms of reduced body weight and accelerated eye opening, which were comparable to previous findings from our laboratory. For adult and middle aged behavioural and endocrine phenotype we observed that the outcome was determined by various interactions between neonatal DEX treatment and H.

Effects of dexamethasone treatment and handling work in same direction

For some characteristics of the animal's phenotype the effects of DEX treatment and H point in the same direction. SAL NH animals show an extremely high initial ASR which is substantially lower in both DEX-treated and H animals. Interestingly, startle reactivity in SAL NH animals is reduced to levels comparable to the other groups towards the end of the startle protocol, resulting in a high degree of habituation in these animals. All groups show startle habituation, but since initial startle was lower in DEX-treated and H animals, the degree of habituation is also lower in these groups. Overall, both DEX and H appear to reduce acoustic startle reactivity. A similar phenomenon has been shown in previous studies from our group (Claessens et al, unpublished), however different control groups were used. Others have also reported that H animals show reduced ASR compared to NH individuals (22). The effects of neonatal glucocorticoid treatment on ASR have not

been frequently studied, but Ferguson and colleagues showed no effect of DEX treatment on postnatal day 7 on adult ASR (23).

The impact of DEX treatment and H on CORT responsiveness follows a comparable pattern: both DEX treatment and H enhance negative feedback of the HPA axis, leaving SAL NH animals with a significantly prolonged CORT response. A suppressed CORT response following acute restraint - and other types of - stress in adult rats that were neonatally exposed to DEX has been reported before (12, 24), as well as the impact of H leading to enhanced stress recovery (19, 25). Although functional outcome is similar, the effects of DEX treatment and H are likely to be mediated via different mechanisms. H animals show increased expression of GR compared to NH animals (26), whereas such an effect has never been shown in response to postnatal DEX treatment. However, prenatal DEX treatment has been reported to result in an increase in hippocampal GR density (27).

Overall it can be concluded that SAL NH animals display a 'reactive' phenotype. As expected, the experience of H reduces this reactivity. Interestingly, neonatal exposure to glucocorticoids has a comparable effect.

Dexamethasone treatment interacts with handling

H enhanced PPI in SAL treated animals, but did not affect DEX treated animals. Although Pryce and colleagues did not report differences in PPI after neonatal H (22), the current PPI enhancing effect of H is in line with previous findings from our laboratory (chapter 4 of this thesis), although in that study the interaction with DEX treatment was not investigated. Zhang and colleagues have reported enhanced PPI in animals receiving high compared to low levels of maternal care during infancy (28). This is in line with our observation that maternal care is enhanced upon reunion following H. Neonatal glucocorticoid treatment has resulted in inconsistent findings regarding PPI phenotype. Ferguson and colleagues report no effect of DEX treatment (23) whereas Hauser and colleagues report an increase in PPI after prenatal DEX, which was not replicated (29). Our data indicate no overall effect of DEX treatment on PPI. However, DEX treatment appears to reduce the susceptibility to H effects.

A comparable type of interaction was observed for spatial learning in the water maze at middle age: DEX treatment altered performance of NH animals, whereas in H animals no effect of DEX treatment was observed. Several studies have shown the adverse effects of neonatal DEX treatment on water maze performance (8). We observed that DEX NH animals need significantly less time to find the platform compared to SAL NH animals, especially on the first day of training. Brabham and colleagues also showed that animals exposed prenatally to DEX (although raised by a non DEX-treated foster mother) perform better than other (SAL-treated) groups in the water maze (27). These findings were unexpected and the authors have suggested an important role for postnatal maternal care which might be different in mothers exposed to DEX or vehicle during pregnancy.

Similarly, there might have been differences in maternal care directed towards DEX vs SAL offspring in our current study. Since we observed maternal care

directed towards the entire litter containing both SAL and DEX treated offspring, we cannot be sure whether treatment-specific differences exist and/or contribute to the adult phenotype. Although H decreases the impact of DEX treatment, we did not find an overall effect of H on water maze performance. While H has been shown to improve spatial learning in the water maze, an effect that lasts up to old age (20, 30), our data are in line with those from several other studies which do not indicate improved circular maze performance in H compared to NH animals (25, 31, 32).

Neonatal glucocorticoid exposure reduced the sensitivity to the beneficial effects of H on PPI, whereas H reduced the sensitivity to the beneficial effects of DEX treatment on spatial learning.

Handling compensates for dexamethasone-induced effects

A different interaction between DEX treatment and H was observed for spatial orientation in the T-maze. DEX NH animals do not discriminate between the new and familiar arm upon re-exposure to the same extent as other groups do, in favour of the new arm. This DEX-induced effect can be reversed by H. The impact of neonatal H on T-maze performance has been reported before (33) and appears to result in a higher discrimination rate compared to NH animals, especially with increasing age (25). The impact of neonatal DEX treatment on T-maze learning has, to our knowledge, not been described.

Spatial orientation with a long-term memory component is affected by DEX treatment and can be fully restored by neonatal H.

Handling effect – no dexamethasone effect

Not all behaviours are affected by both DEX treatment and H. For the behavioural phenotype observed in the fear conditioning paradigm, we found that immediate reactivity, in terms of freezing, to the foot shock was higher in NH compared to H animals. NH animals continue to show more freezing during re-exposure to the shock context 24h later. Although H has been reported to enhance contextual fear conditioning (34), our findings cannot be interpreted as a difference in contextual fear learning or memory, due to differences in responsiveness directly following shock exposure, and are more likely to indicate a difference in coping style (35). We do not report a main effect of, or an interaction with DEX treatment, which is in line with findings from Kamphuis and colleagues (12).

CONCLUSIONS

We report that the outcome of neonatal DEX treatment was determined by interactions with the effects of H. Overall, SAL NH animals appear to be 'challenged' the most during behavioural testing in adulthood. They show: (1) extreme startle reactivity, (2) low PPI, (3) high freezing in response to a foot shock (although similar to DEX NH), (4) low negative feedback of the HPA axis in response to acute stress, and (5) impaired spatial learning in the water maze. As expected, neonatal

H reduced startle reactivity and enhanced glucocorticoid feedback of the HPA axis. Neonatal DEX treatment, although expected to have detrimental effects on phenotype, resulted in effects comparable to H for several of the parameters studied. DEX treatment led to reduced ASR, enhanced feedback of the HPA axis and improved performance in the water maze. Whereas H reduced the impact of DEX in the T-maze and the water maze, DEX treatment reduced the sensitivity for H effects as observed for PPI.

These findings clearly show that the outcome of neonatal DEX-treatment: 1) is not deterministic, 2) highly depends on other characteristics of the postnatal environment, and 3) is potentially mediated by alterations in mother-pup interaction. Moreover, they highlight the importance of interaction between individual components of the early postnatal environment.

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CHAPTER 6

General Discussion

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The aim of the research described in this thesis was to explore, using a rat model, the short- and long-term consequences of 1) very subtle differences in early-life experience, induced by within-litter variation in individual pup-directed maternal care, and 2) pharmacological manipulation of the neonate's brain and HPA axis development by early synthetic glucocorticoid exposure. The latter treatment, used clinically to treat respiratory distress syndrome in prematurely born infants, has been reported to result in adverse neurodevelopmental outcomes. Therefore, we investigated the possibility to reverse these frequently reported detrimental effects using both pharmacological and behavioural interventions.

In this chapter, the experimental findings described in this thesis will first be summarized and evaluated. Then, the implications of the findings for the field of developmental programming will be discussed, as well as the impact of potential intervention strategies to prevent the adverse effects of dexamethasone treatment on the developing brain. The chapter is concluded with an adjustment of our initial hypothesis on the intensity of individual components of the early postnatal environment, and their potential to program later-life phenotypes.

SUMMARY OF FINDINGS

Within-litter differences in maternal care

To investigate the existence of subtle individual differences in maternal environment we used an adapted version of a frequently used model of naturally occurring variations in maternal care (1). This allowed the study of within-litter differences in single pup-directed maternal licking and grooming in Wistar rats to test the hypothesis that maternal care is equally distributed across littermates.

We reported that maternal care is not homogeneously distributed across littermates. Thus, besides differences in maternal care between litters, also variation within the litter exists.

Next, the endocrine response to an acute novelty stressor was investigated during adolescence and adulthood to test the hypothesis that within-litter differences in maternal care have long-lasting impact on stress phenotype.

We reported that these very subtle differences in early-life experience have long-lasting impact on the offspring's later-life endocrine stress phenotype, although these findings were biased by a gender-preference displayed by the majority of dams.

Neonatal glucocorticoid exposure

To investigate the impact of neonatal exposure to synthetic glucocorticoids, Long Evans rat pups were injected with dexamethasone according to a protocol based on the treatment regimen for premature infants used in clinical settings. Preterm infants are mostly treated with glucocorticoids between week 26 and

32 of gestation. Therefore, we aimed to expose rats to dexamethasone during a period in which the stage of brain development is comparable to that of a human fetus during the last trimester of pregnancy (2).
We first investigated:

1. the acute consequences of this treatment on cell proliferation and glial activity in the developing brain
2. the possibility to prevent these alterations by pharmacological intervention using local GR antagonist administration

We reported that, as expected, neonatal glucocorticoid exposure acutely affects hippocampal cell proliferation and number of glial cells in the developing brain. These short-term alterations can be partially prevented by central GR antagonist pre-treatment, which was suggested as a potential intervention strategy to protect the developing brain against the detrimental effects of glucocorticoid exposure.

Then, we investigated the consequences for postnatal development, as well as the adult, middle aged and aging endocrine and behavioural phenotype.

We reported that although neonatal dexamethasone treatment leads to developmental alterations, the frequently reported adverse effects on adult endocrine and behavioural phenotype were not observed. Based on the data it was suggested that – contrary to our expectations - daily handling of the neonate during the postnatal period modulates - and potentially overrides - the outcome of neonatal dexamethasone exposure.

Finally, we tested the hypothesis that brief daily separations from the nest, leading to an 'enriched' postnatal environment due to enhanced maternal care upon reunion with the dam, modulate the negative programming effects of neonatal glucocorticoid exposure.

We reported that the effects of neonatal dexamethasone treatment interact with those of neonatal handling in shaping the adult endocrine and behavioural phenotype (see table 1). We observed that non-handled animals appear to be more susceptible to the impact of neonatal dexamethasone treatment, compared to handled animals. These findings emphasize that the outcome of neonatal glucocorticoid exposure is not deterministic and strongly interacts with other components of the postnatal environment.

GLUCOCORTICOIDS DURING DEVELOPMENT

In **chapter 3** the acute effects of neonatal dexamethasone treatment on markers for brain development are described. Our findings, a reduction in growth, hippocampal cell proliferation and number of GFAP-positive cells in the hilus and corpus callosum,

Table 1. Overview of dexamethasone- and handling-induced effects on adult endocrine and behavioural phenotype.

	PPI	ASR	CHB	T-maze	FA	MWM	HPA
DEX	=	↓	↑	↓	=	↑	↓
H	↑	↓	?	↑	↓	↑	↓
DEX x H	+	+	?	+	-	+	+

DEX: dexamethasone, H: handling, DEX x H: dexamethasone x handling interaction. PPI: Pre-pulse inhibition, ASR: acoustic startle reactivity, CHB: circular hole board, FA: fear acquisition (freezing response to foot shock), HPA: CORT response to restraint stress, MWM: morris water maze. ↑: increase, ↓: decrease, =: no increase/decrease, ?: not studied, +: interaction, -: no interaction.

followed our expectations based on numerous studies investigating the impact of early glucocorticoid exposure on neuronal and glial proliferation.

The role of glucocorticoids during brain development has been studied for decades, starting long before their use in treating respiratory disease of premature infants (3). Appropriate levels of glucocorticoids are necessary for normal development (4-6). Absence of glucocorticoids threatens survival as has been demonstrated in glucocorticoid-insufficient and GR-deficient animals, which die of respiratory failure due to abnormal lung development (7, 8). During normal pregnancy the activity of the maternal HPA axis changes dramatically, leading to increased circulating glucocorticoid levels in late gestation (9). This rise in glucocorticoids is essential for maturation of various tissues (10). Therefore, premature infants, or pregnant women at risk of giving premature birth, are treated with synthetic glucocorticoids to stimulate lung maturation.

However, not only absence of glucocorticoids, but also elevated levels can disturb normal development. This happens in case of exaggerated levels of *endogenous* glucocorticoids, due to maternal stress during pregnancy. Exposure to *synthetic* glucocorticoids such as dexamethasone is considered even more dangerous since those are not converted by placental 11β HSD2 (11, 12), making them more likely to cross the placenta and enter the fetal circulation (13). Additionally, synthetic corticosteroids show little affinity for corticosteroid binding globulin and can readily enter the brain, since the blood-brain barrier is not fully developed in neonates (14, 15).

Neonatal glucocorticoid exposure suppresses growth and proliferation

Growth retardation, like we observed in dexamethasone-treated animals, is one of the frequently reported side effects of exposure to increased levels of glucocorticoids in early-life. This phenomenon is probably the consequence of a glucocorticoid-induced (transient) catabolic state and suppression of insulin-like growth factor (IGF)-axis activity (16, 17).

Besides somatic growth, brain growth is also retarded in response to excessive glucocorticoid exposure. Exogenous glucocorticoid treatment results in reduced cerebral weight, DNA content and cell proliferation (18-21). Especially

hippocampal cell proliferation has been shown to be sensitive to the effects of neonatal glucocorticoid exposure, likely because it is one of the areas undergoing substantial postnatal growth (4). Whereas proliferation in this area is suppressed *during* exposure, a rebound effect is observed upon *cessation* of treatment leading to 'normal' dentate gyrus volume in adulthood (18). This early finding in rodents was later confirmed by studies in the marmoset (22, 23) and also matches the findings reported in **chapter 3** of this thesis. These findings indicate the amazing plasticity and capacity for recovery of the neonatal brain.

Long-lasting implications of acute transient effect

Glucocorticoid exposure enhances overall maturation and differentiation at the expense of growth and proliferation (10). This effect is either beneficial or detrimental depending on the developmental context. A premature system might benefit from a cue that stops growth and enhances differentiation, since tissue maturation rather than growth is necessary for survival in the extra-uterine environment. Thus, the acute benefits for survival are obvious. And although rebound effects, normalizing the acute proliferation-inhibiting effect, have been reported in various studies, it is likely that there will be consequences for later-life functioning.

In rodents, the development of the hippocampus, especially the dentate gyrus, happens largely postnatally (24-27). It is believed that the extensive plasticity that characterizes normal early development is necessary for proper adult functioning of the hippocampus (27-29). Even a transient reduction in proliferation during a developmental stage that is normally characterized by high levels of proliferation, can influence adult functioning of the hippocampus and connected structures. These alterations may contribute to the frequently reported cognitive impairments observed after neonatal glucocorticoid exposure (30, 31).

Effects on different cell populations

Besides a reduction in cell proliferation on postnatal day 4, we observed a substantial reduction in the number of astrocytes on postnatal day 10. Because the timing of glucocorticoid treatment in the preterm infant (and also in our animal model) coincides with a period of substantial glial proliferation, it is not surprising that gliogenesis is affected. Indeed, studies performed in the 1980's showed reductions in myelination in postnatally treated rats, likely due to reduction in oligodendrocytes number (32). This effect was later supported by evidence in other species (33, 34). Interestingly, Bohn and Friedrich reported - similar to their neuronal observations - a rebound effect with significantly enhanced genesis of oligodendrocytes after termination of glucocorticoid treatment. We did not report such a rebound effect for the number of GFAP-labelled cells, at least not after a 1 week recovery period. Tsuneishi and colleagues also reported that GFAP levels were still reduced 10 days post-treatment, but normalized to control levels 20 days post-treatment (35), indicating that the glial rebound process needs more time and likely proceeds between postnatal days 10 and 20.

Protecting the brain

The acute effects after neonatal dexamethasone treatment described in **chapter 3** of this thesis can be partially prevented by centrally blocking the GR prior to treatment. Beneficial effects of systemic GR blockade prior to neonatal dexamethasone treatment on hippocampal functioning have been reported previously (36). Additionally, the effects of chronic corticosterone treatment and of chronic stress in adulthood on hippocampal structure and function appear reversible upon a short course of GR antagonist treatment (37, 38).

The findings described in **chapter 3** of this thesis are to our knowledge the first report of beneficial effects of brain-specific GR blockade, which is more clinically relevant given the beneficial peripheral effects of dexamethasone treatment on lung development in prematurely born infants. Therefore, central GR blockade might serve as a potential pharmacological intervention strategy for adverse dexamethasone-induced effects. However, the invasive way of antagonist administration might have functional consequences as a result of astrogliosis induced by the intracerebroventricular injection as observed in our studies. Therefore, future studies using another, less invasive, route of administration, such as intrathecal injection, are needed to investigate the clinical potential of this intervention strategy.

Window of vulnerability

The specific developmental outcome of neonatal glucocorticoid exposure, including the potential for recovery, depends on numerous factors such as dose and timing of exposure, as well as the specific brain area studied. The impact is probably most considerable in brain areas undergoing growth and development at the time of exposure, such as the cerebellum and hippocampus in the postnatal rodent brain (4, 21). However, these brain areas still display a distinct postnatal developmental trajectory.

The growth spurt of the external granular layer of the cerebellum occurs slightly later compared to that of the granule layer in the dentate gyrus. Therefore, glucocorticoid treatment on postnatal days 7-18 has a more severe impact on cerebellar development compared to treatment on days 1-4, which has a large impact on dentate gyrus development (19). The decrease in cerebellar granule cell proliferation never fully recovers after glucocorticoid treatment, in contrast to the dentate population. These findings show that even small variations in timing of glucocorticoid exposure can result in targeting different areas and functions, and that certain areas are apparently more resilient to the growth suppressing effects of glucocorticoids.

Although, timing of treatment in the clinical situation is - and should be - mostly driven by severity of prematurity-associated pathology, research on the mechanism underlying the adverse neurodevelopmental effects using animal models should consider the sequence of events during brain development and choose time of treatment accordingly.

CONSEQUENCES OF NEONATAL GLUCOCORTICOID EXPOSURE FOR ADULT PHENOTYPE

Besides studies on the acute effects, numerous reports have been published on the long-lasting impact of neonatal dexamethasone treatment in rats.

Health- and lifespan

Probably the most striking finding is that neonatal dexamethasone treatment results in a significant shortening of the lifespan (39-41). Liu and colleagues reported survival rates of 79% and 83% at 50 weeks of age after neonatal dexamethasone treatment, compared to 100% survival in saline-treated controls at the same age. A 50% reduction in the dexamethasone dose leads to survival rates comparable to those of saline-treated animals. This shortening of the lifespan was suggested to be the result of renal failure, since the surviving dexamethasone-treated animals displayed increased blood pressure, kidney damage and urinary protein content. These effects might be related to an early inflammatory response in the kidney. The authors observed that on postnatal day 2, TNF-α gene expression was suppressed in the kidney, followed by a significant increase on day 7 (41).

Additionally, Kamphuis and colleagues reported an overall shortening of lifespan of 25% in male rats neonatally treated with dexamethasone, with a mean survival of 21 months in dexamethasone- vs 29 months in saline-treated animals (39). These findings were associated with cardiac and renal failure already present at 15 months of age. Moreover, dexamethasone-treated animals already showed hypertension in young adulthood. The effects on the heart were in line with earlier findings of progressive hypertrophic cardiomyopathy (42) which might be the result of acute suppression of cardiomyocyte proliferation observed in the rat pup in response to glucocorticoid treatment (43). Additionally, an acute reduction in mitotic activity in the renal cortex after dexamethasone treatment results in lower nephron numbers and renal damage in later-life (44).

Neonatal dexamethasone treatment also alters adult immune function. An increase in severity and incidence of inflammatory autoimmune disease in adulthood has been shown in adult animals, treated as infants with dexamethasone. These animals also showed a reduced corticosterone response to LPS challenge. Additionally, LPS-stimulated macrophages of these animals showed an altered immune profile in terms of reduced TNF-α and IL-1β production (45). Dexamethasone-treated animals also displayed differential long-term effects on the expression of Vβ genes in CD4 and CD8 splenocytes which were preceded by changes in intrathymic corticosterone production and in CD4/CD8 thymocyte ratio (46).

As also extensively discussed in **chapter 4 and 5** of this thesis, there are reports on impaired spatial learning (30) and hippocampal synaptic plasticity (30, 47) in adulthood which appear to be in line with the acute effects of dexamethasone treatment on hippocampal cell proliferation. Moreover, many studies have demonstrated alterations in endocrine responsiveness to stress (48-50).

As described in **chapters 4 and 5**, the adult phenotype of animals neonatally exposed to dexamethasone observed in our studies did not follow the expectations based on this substantial amount of data showing a severely affected adult phenotype. For instance, we did not report such dramatic effects of early glucocorticoid treatment on survival, although the steroid was administered in the same dose and during the same postnatal days as in the studies of Kamphuis and Liu. Although our dexamethasone-treated animals appeared to show a steeper survival curve, this effect did not reach statistical significance at 25 months, an age at which effects were detectable in the studies mentioned above.

Our findings indicate that the outcome of early glucocorticoid exposure is not deterministic and might depend on interactions with multiple internal (genetic, developmental stage) and external (environmental) factors, which might contribute to this apparent inconsistency.

Genetic factors

Opposing effects, in terms of body weight, motor performance and social interaction, after early hydrocortisone treatment, have been reported in closely related species (pine and meadow voles) (51). Following these findings, it can be suggested that also more subtle genetic differences might contribute to variation in the outcome. The majority of rat studies are performed in Wistar and Sprague-Dawley rats, in contrast to Long-Evans rats used in our experiments. In studies using mice it has been shown that certain strains are more sensitive compared to others, to the programming effects of maternal care received in infancy, with consequences for later-life drug self-administration and depression-related behaviours (52). Additionally, there are substantial inter-strain differences in expression of corticosteroid receptors and sensitivity of these receptors to their ligands (53, 54), which makes certain strains react differently to programming effects of glucocorticoids compared to others.

Timing of exposure

As reported before regarding acute effects on brain development, slight differences in the timing of treatment can have major impact on later-life phenotypes as well. Variation of only days can result in targeting either mostly cognition-related functions in the dentate gyrus or motor-related circuits in cerebellum. More specifically, Meaney and colleagues reported that there is a critical period for glucocorticoid-induced disruption of play-fighting behaviour, with substantial effects after corticosterone treatment on postnatal days 1-4, but not after treatment on days 9-10 (55).

Interestingly, our treatment protocol is identical to that used by Kamphuis and colleagues, who have described a broad spectrum of molecular and behavioural alterations after neonatal dexamethasone treatment on postnatal days 1-3 (30, 39, 50, 56). Although our dose and timing of dexamethasone treatment is identical to theirs, substantial differences exist in functional outcome in adulthood.

Impact of experimental design

An important difference between these protocols lies in the litter composition, meaning the use of either a 'split-litter', with dexamethasone and control (saline) treated animals within one litter, or a 'whole-litter' design, in which all pups in the litter are subjected to the same treatment. We have chosen the split-litter design because we aimed to distribute equally over the different treatment groups: 1) genetic differences between litters, and 2) differences in maternal care between dams.

Kamphuis and colleagues however report that the use of a split-litter design might enhance the effects of neonatal dexamethasone treatment on various developmental parameters (57). The authors show that maternal care is not different towards dexamethasone-treated compared to saline-treated offspring, as investigated using a whole-litter approach. They suggest that the amount of maternal care will be lower towards dexamethasone- compared to saline-treated pups when they are reared together in the same litter, due to competition of the pups for maternal care, leading to the enlargement of the dexamethasone effect. This within-litter effect was unfortunately not investigated. However, as we described in **chapter 2** of this thesis, these within-litter differences in maternal care can be substantial.

Maternal mediation

Interestingly, rather than *more* substantial differences, we observed *less* differences between saline and dexamethasone-treated animals using a split-litter design (**chapter 4**), compared to Kamphuis' whole-litter reared animals. We suggested this to be the result of the brief daily periods of separation from the nest, necessary to mark the pups for identification between saline- and dexamethasone-treated littermates. This neonatal handling is known to result in alterations in HPA axis responsiveness (58-61) and cognitive performance (62) in adulthood. The effects of handling are suggested to be mediated via enhancing maternal care (63). Therefore, we suggested that the absence rather than enlargement of differences between saline and dexamethasone-treated offspring (**chapter 4**) are the result of enhanced maternal care directed towards *all* handled pups.

Indeed, we showed in **chapter 5** that maternal care was enhanced in handled compared to non-handled animals, a finding that supports our hypothesis. Unfortunately, in this study we only observed whole-litter oriented maternal care since we aimed to compare maternal care levels towards handled vs non-handled animals. The study of differences in individual pup-directed maternal care requires marking of individual pups for identification (like we did in **chapters 2** and **4**), which would have interfered with the undisturbed environment of the non-handled animals in **chapter 5**. Therefore, future studies using a different type of marking are needed to investigate the presence of within-litter differences in maternal care directed towards dexamethasone- and saline-treated pups.

If there is competition between pups in favour of saline-treated offspring, to such an extent that a lack of maternal care enhances the dexamethasone-induced

phenotype (as suggested by Kamphuis and colleagues), this effect is apparently blunted when pups are daily handled and maternal care levels are increased to such an extent, that they override differences induced by neonatal dexamethasone treatment, as was observed in **chapter 4**. We did observe in **chapter** 5 for several cognitive functions (T-maze and water maze) that indeed the impact of neonatal dexamethasone treatment is more substantial in non-handled, compared to handled animals. These findings further support the idea that enhanced maternal care following handling, results in blunting of the dexamethasone effect.

Implications for the clinic

These observations clearly demonstrate that neonatal dexamethasone treatment does not override all other environmental factors, as initially expected. The developmental impact of early glucocorticoid exposure is not deterministic and strongly interacts with other environmental factors. Although the effects of handling did not prevent all dexamethasone-induced effects and the findings cannot be directly extrapolated to the human situation, the observations described in this thesis might be valuable for the clinic. They could create awareness for the important contribution of environmental influences mediating glucocorticoid-induced effects. They emphasize, for instance, the risk of a lack of maternal contact in incubator care preterm infants. Additionally, they contribute to evidence from studies investigating the beneficial effects of massage therapy (64-66) as well as other protocols directed at enhancing maturation in the preterm infant.

It would be interesting and important to investigate whether variation in the degree of care or contact to a caregiver can be an additional factor (besides dose and time of exposure) explaining variation observed in the degree of adversity of the outcome of dexamethasone treatment in human preterm infants. Overall, the findings described in this thesis seem promising for the human situation since they show that besides pharmacological intervention; also behavioural intervention can be effective in mediating the development of certain dexamethasone-induced alterations.

INDIVIDUAL DIFFERENCES IN MATERNAL CARE

As suggested before, within-litter competition for maternal care between pups - potentially induced by pup treatment (67, 68) - might modulate (enhance) the direct impact of such a treatment. Although we did not investigate treatment-specific within-litter effects on maternal care, we did observe that naturally-occurring within-litter differences in maternal care exist (**chapter 2**). It was already known that variation in maternal care has profound and long-lasting impact on adult stress phenotypes (69). This association was however based on differences in maternal care *between* litters, and the assumption that care is equally distributed across littermates, rather than on what is experienced by individual pups *within* the nest.

Previous studies have suggested that pups from the same litter display substantial variation in behavioural phenotype later in life (70). Moreover, it was

previously reported that a 24 hour maternal deprivation results in the amplification of individual differences in stress responsiveness, rather than having a generalized outcome (71). Variation in individual mother-infant interactions during early-life might contribute to the development of this dichotomy and enhance either vulnerability or resilience to the impairing effects of the deprivation.

Here, we reported that *within*-litter differences that are up to 10-fold smaller than those used previously to characterise dams, appear to have predictive value for later-life stress phenotype. Since these effects interacted with gender, the gender-specific effects of within-litter differences in maternal care require further investigation. Additionally, it has been recently shown that hippocampal synaptic plasticity and glucocorticoid receptor mRNA expression are affected by within-litter differences in maternal care (72) to a similar extent as has been shown for between-litter variation in care (73).

Interestingly, in order to investigate individual pup-directed levels of maternal care, it was necessary to daily mark the pups for identification. This procedure obviously resulted in a substantial amount of neonatal handling, the impact of which has been described extensively in **chapters 1, 4 and 5**. The impact of handling is considered substantial and long-lasting. Intriguingly, the extremely subtle within-litter differences in maternal care were apparently not overruled by the supposedly strong overall effect of handling. Although all pups were exposed to handling, differences in endocrine stress responsiveness were still observed in adolescence and adulthood, although in a gender-specific manner.

IMPACT OF INDIVIDUAL COMPONENTS OF THE EARLY-LIFE ENVIRONMENT AND THEIR INTERACTION

This finding showing the impact of very subtle differences in early maternal environment potentially overriding the effects of handling, together with the observation that the consequences of dexamethasone exposure can be strongly mediated by the effects of handling, or handling-induced variation in maternal care, changed our hypothesis about the intensity of the impact of individual aspects of the early-life environment. Thus, although we initially expected that the order of magnitude of the programming effect would be:

Dexamethasone > Handling > Maternal Care

the findings reported in this thesis suggest that the programming impact of early-life experiences more closely resembles:

Maternal Care > Handling > Dexamethasone

Obviously, other components should be taken into consideration, such as (interaction with) genetic background and timing of exposure as described above, as well as the environmental context in which the animal is tested, the impact of which will be discussed below.

CONTEXT MATTERS

We have reported that the outcome of early-life experiences highly depends on the context in which they take place, such as in an undisturbed environment with continuous maternal presence or in a more challenging environment with transient separations from the mother or exposure to novel situations. These factors determine the outcome (and degree of adversity) of for instance neonatal synthetic glucocorticoid exposure.

The environmental context in later-life also mediates the impact of early experiences. The 'predictive adaptation plasticity hypothesis' (74-78) is based on the concept that a developing organism responds to cues (e.g. maternal care, glucocorticoids) in its environment by changing certain aspects of its homeostatic regulation (e.g. HPA axis) in order to produce a phenotype that is highly adapted to its current environment, assuming that this environment is comparable to its future environment. Following this reasoning, a high degree of 'mismatch' between the early- and later-life environments might account for an increased risk to develop diseases in adulthood (75-78).

More specifically, the outcome of early maternal environment on later cognitive performance seems dependent on later-life environmental context (73, 79), with adult offspring exposed as neonates to low levels (or temporary deprivation) of maternal care showing poor cognitive performance in a low-stress context (80). However, in a high-stress context their performance was better as compared to that of animals that received high levels of maternal care, which were actually impaired under these stressful conditions (73, 79). These findings suggest that the influence of environmental experiences during development might serve as a basis for resilience to stressful challenges in later-life.

Although the concept of 'matching environments' was not explicitly investigated in the studies described in this thesis, the potential beneficial long-term outcome after neonatal dexamethasone exposure, preparing the organism for better coping with stressful events in adult life, should be taken into consideration in the design of future studies.

CONCLUDING REMARKS

We investigated the impact of neonatal dexamethasone exposure, which we expected to overrule any other environmental effect. Interestingly we observed that dexamethasone-induced effects interact strongly with other environmental factors during development and are therefore susceptible to intervention strategies.

We have indications that extremely subtle within-litter differences in maternal care have long-lasting impact on later-life stress phenotypes, potentially overriding the supposedly robust effects of handling. These findings indicate that all components of the postnatal environment, no matter how subtle, interact in shaping the adult phenotype.

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SUMMARY

SUMMARY

It is well documented that early-life experiences are involved in shaping later-life phenotypes. Both human and animal studies have reported the impact of (adverse) early experiences on the development of the stress system, and its consequences for the development of stress-related disorders. The aim of the research described in this thesis was to explore the acute and long-lasting consequences of two distinct types of postnatal experience, varying substantially in nature and severity. In one study, the outcome of very subtle differences in maternal environment was examined. In another series of experiments, exposure to synthetic glucocorticoids during the early postnatal period was investigated. Furthermore, two potential intervention strategies to prevent the frequently reported adverse effects of glucocorticoid-induced disruption of normal development and brain maturation were evaluated.

In **chapter 1** an overview was given of important concepts for the study of developmental programming. Additionally, several animal models used in experiments described in this thesis were introduced.

In **chapter 2** we tested the hypothesis that maternal care is equally distributed across littermates and results in the development of a uniform stress phenotype with the litter. To investigate the distribution of maternal care across individual pups within the litter, we used an adapted version of a frequently used model of naturally-occurring variations in maternal care (Champagne et al. 2003). This allowed the study of within-litter differences in single pup-directed maternal licking and grooming. We reported that maternal care is not homogeneously distributed across littermates. Thus, besides differences in maternal care between litters, also variation within the litter exists. Next, the endocrine response to an acute novelty stressor was investigated at adolescence and adulthood in pups receiving low versus high levels of maternal care within the litter. We reported that these very subtle differences in early-life experience have long-lasting impact on the offspring's later-life endocrine stress phenotype. Interestingly, we observed that rat mothers show a preference for male over female pups, resulting in a bias in the distribution of males and females over the low and high maternal care group. Therefore, future studies are needed to investigate the gender-specific impact of within-litter differences in maternal care on later-life stress phenotype.

In **chapter 3** we investigated the acute consequences of neonatal glucocorticoid treatment on cell proliferation and glial activity in the developing brain. To investigate the impact of neonatal exposure to synthetic glucocorticoids, Long Evans rat pups were injected with dexamethasone on postnatal day 1, 2 and 3, according to a protocol based on the treatment regimen for premature infants used in clinical settings. We reported that neonatal glucocorticoid exposure acutely, but transiently reduced hippocampal cell proliferation. Additionally, the number of glial cells in the corpus callosum and hippocampus was reduced in dexamethasone-treated animals one week post-treatment. These short-term alterations in the developing brain might contribute to the frequently reported detrimental impact of neonatal dexamethasone treatment on adult phenotype. Next, we tested the possibility to prevent these alterations by pharmacological

intervention using central GR antagonist administration prior to dexamethasone treatment. We reported that although central mifepristone administration did not prevent the reduction in hippocampal cell proliferation, the dexamethasone-induced reduction in number of glial cells was fully normalized by central mifepristone pre-treatment, which was suggested as a potential intervention strategy to protect the developing brain against certain detrimental effects of glucocorticoid exposure.

Then, we investigated in **chapter 4** the consequences of this treatment for postnatal development, as well as the young adult, middle aged and senescent endocrine and behavioural phenotype, in order to test the hypothesis that neonatal glucocorticoid exposure results in long-lasting alterations in endocrine and behavioural reactivity as well as in a shortening of the lifespan. We reported that neonatal dexamethasone treatment leads to developmental alterations, such as growth retardation and accelerated eye opening. The frequently reported adverse effects on adult endocrine and behavioural phenotype were however not observed. Neonatal dexamethasone treatment did not result in spatial learning impairments, nor did it result in altered stress-induced HPA axis activation and acquisition of fear. Additionally, we did not report a shorter lifespan in dexamethasone- compared to saline-treated animals. It was suggested that - contrary to our expectations - postnatal handling of the neonate, as a result of our within-litter treatment design, modulates - and potentially overrides - the outcome of neonatal dexamethasone exposure. Indeed, additional studies showing reduced pre-pulse inhibition, motor performance and spatial learning, as well as prolonged stress-induced HPA axis activation in animals that experienced a totally undisturbed postnatal environment compared to handled animals (both saline and dexamethasone treated), strengthen this hypothesis, which was finally tested in **chapter 5**.

In **chapter 5** we investigated whether neonatal handling could serve as a second - behavioural - intervention strategy to rescue the dexamethasone-induced phenotype. We reported that neonatal handling enhanced maternal care upon reunion, and that the effects of handling indeed interacted with those of neonatal dexamethasone treatment in shaping the adult endocrine and behavioural phenotype. Although dexamethasone treatment and handling yielded comparable effects on the restraint stress-induced CORT response and acoustic startle reactivity, acquisition of fear was only affected by handling without an effect of dexamethasone exposure. Interestingly, it could be concluded that dexamethasone treatment reduced the sensitivity for beneficial effects of handling, as was observed for pre-pulse inhibition. Additionally, non-handled animals appeared to be more susceptible to the impact of neonatal dexamethasone treatment compared to handled animals, as was demonstrated for spatial learning in the water maze. Moreover, the impairing effects of dexamethasone treatment in spatial orientation in the T-maze were only observed in non-handled animals. Overall it appears that dexamethasone-treatment mostly affects non-handled animals, specifically for tasks with a cognitive component.

These findings emphasize that the outcome of neonatal glucocorticoid exposure is not deterministic and strongly interacts with other components of the postnatal environment.

In **chapter 6**, the experimental findings described in this thesis were summarized and evaluated, and the implications for the field of developmental programming were discussed. We concluded that dexamethasone-induced effects interact strongly with other environmental factors during development and are therefore susceptible to intervention strategies, contrary to our expectations that the impact of neonatal dexamethasone exposure would overrule any other environmental effect. These observations might be valuable for the clinic, creating awareness for the important contribution of environmental influences, such as the degree of contact to the caregiver, in mediating glucocorticoid-induced effects.

Additionally, the finding that very subtle within-litter differences in maternal care have long-lasting impact on later-life stress phenotypes, highlights the important contribution of all components of the postnatal environment, no matter how subtle, in shaping the adult phenotype.

SAMENVATTING

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Vroege levenservaringen dragen bij aan verschillende aspecten van de ontwikkeling van een individu. Er zijn veel gegevens van studies in dier en mens die aangeven dat stressvolle gebeurtenissen vroeg in het leven invloed hebben op de ontwikkeling van het stress syteem en daardoor mogelijk bijdragen aan het ontstaan van stress-gerelateerde aandoeningen. Het doel van het onderzoek beschreven in dit proefschrift was de korte en lange termijn effecten te beschrijven van twee soorten vroege levenservaringen die van elkaar verschillen in type en intensiteit. Door middel van diermodellen onderzochten we de consequenties van: 1) zeer subtiele verschillen in moederzorg tijdens de eerste week van het leven (**hoofdstuk 2**), en 2) blootstelling aan een synthetisch glucocorticoïde tijdens de eerste 3 dagen van het leven (**hoofdstukken 3, 4 en 5**). Aangezien deze laatste ervaring, een veelgebruikte behandeling voor respiratoire aandoeningen in prematuren, negatieve gevolgen lijkt te hebben voor de neurologische ontwikkeling, hebben we tevens de mogelijkheid onderzocht om deze negatieve effecten tegen te gaan danwel te verminderen door toepassing van twee interventie strategiën.

In **hoofdstuk 1** werden verschillende concepten geïntroduceerd die belangrijk zijn voor onderzoek op het gebied van 'developmental programming'. Daarnaast hebben we enkele relevante diermodellen besproken, waarvan sommige ook gebruikt werden in de experimenten beschreven in dit proefschrift.

In **hoofdstuk 2** hebben we allereerst de hypothese getest dat moedergedrag gelijk verdeeld wordt over individuele pups binnen het nest. Om deze hypothese te toetsen hebben we een aangepaste versie van een bestaand model gebruikt om moedergedrag in ratten te bestuderen. In tegenstelling tot het originele model, was het in het aangepaste model mogelijk om individuele moeder-pup interacties te bestuderen en scoren. We concludeerden dat moedergedrag niet homogeen is verdeeld over de verschillende pups in het nest. Naast eerder aangetoonde verschillen tussen nesten (en dus moeders) blijkt er ook variatie te bestaan in de hoeveelheid moedergedrag ontvangen door individuele pups binnen een nest. Vervolgens hebben we de hormonale stress reactie gemeten in de adolescentie evenals op volwassen leeftijd in dieren die veel danwel weinig moedergedrag hadden gekregen in vergelijking met hun broers en zussen. We lieten zien dat subtiele verschillen in moederzorg, ontvangen tijdens de eerste week van het leven, leiden tot variatie in stressgevoeligheid later in het leven. Opvallend was dat de moeders een voorkeur leken te hebben voor de mannelijke in vergelijking met de vrouwelijke pups. Dit zorgde voor een ongelijke verdeling van mannelijke en vrouwelijk dieren over de 'veel' en 'weinig' moedergedrag groepen. Vervolgonderzoek naar de precieze, sexe-specifieke, effecten van moedergedrag op latere stress gevoeligheid, is dus nodig.

In **hoofdstuk 3** hebben we de korte termijn effecten van blootstelling aan het synthetische glucocorticoïd dexamethason bestudeerd op de ontwikkeling van de hersenen. Meer specifiek hebben we gekeken naar celproliferatie en de hoeveelheid en activiteit van gliacellen. Om de invloed van blootstelling aan glucocorticoïden vroeg in het leven te onderzoeken hebben we Long Evans

ratten geinjecteerd met dexamethason op dag 1, 2 en 3 na de geboorte. Dit is gebaseerd op het behandelingsschema dat in medische centra gebruikt wordt om longrijping te stimuleren na vroeggeboorte. We zagen dat celproliferatie acuut, maar tijdelijk, was afgenomen in de hippocampus van dieren behandeld met dexamethason. Daarnaast bleek het aantal gliacellen in het corpus callosum en de hippocampus significant te zijn verminderd 1 week na de laatste dexamethason injectie. Deze korte termijn effecten op de ontwikkelende hersenen zouden kunnen bijdragen aan de negatieve bijwerkingen van dexamethason-behandeling op de cognitieve vaardigheden later in het leven, die vaak zijn beschreven in de literatuur. Vervolgens hebben we de mogelijkheid onderzocht om deze effecten op de hersenen te blokkeren door een glucocorticoïd receptorantagonist lokaal in de hersenen toe te dienen vlak voor dexamethason-behandeling. We zagen dat, hoewel de effecten op celproliferatie niet werden beïnvloed, behandeling met de antagonist de afname in aantal gliacellen tegenging. Op basis hiervan zou deze voorbehandeling als potentiele interventie strategie kunnen dienen om de effecten van dexamethason op de hersenen (gedeeltelijk) tegen te gaan.

In **hoofdstuk 4** hebben we de lange termijn effecten van dexamethason-behandeling onderzocht. We hebben gekeken naar de postnatale ontwikkeling, en naar het functioneren van de dieren op jongvolwassen, middelbare en hoge leeftijd om de hypothese te toetsen dat behandeling met dexamethason vroeg in het leven resulteert in lange termijn effecten op cognitieve vaardigheden en stressgevoeligheid, en daarnaast in een kortere levensduur. We zagen dat dexamethason-behandeling, zoals verwacht, resulteerde in een verstoorde postnatale ontwikkeling. Behandelde dieren vertoonden een groeiachterstand en juist een versnelde opening van de ogen. De veel beschreven negatieve lange termijn effecten op het cognitieve en endocriene functioneren op volwassen leeftijd, zagen we echter niet in onze dieren. Dexamethason-behandelde dieren vertoonden geen leerproblemen en hadden ook een normale stressreactie. Ook het effect op de levensduur werd niet waargenomen: wij zagen geen significante verkorting van de levensduur.

We hebben toen onderzocht of het hanteren van de dieren tijdens de postnatale periode (dagelijks 15 minuten scheiden van de moeder, noodzakelijk om individuele dieren te kunnen markeren voor identificatie), de effecten van dexamethason-behandeling zou hebben kunnen beïnvloeden. We hadden verwacht dat het effect van dexamethason-behandeling elke andere manipulatie tijdens de postnatale periode zou overtreffen. Naar aanleiding van de bevindingen in **hoofdstuk 4** hebben we deze hypothese echter herzien. Aanvullende studies in **hoofdstuk 4** laten zien dat dieren die tijdens de postnatale periode volledig met rust gelaten werden verminderde pre-puls inhibitie, motorische en cognitieve vaardigheden hadden, en daarnaast een minder goede negatieve feedback van de endocriene stressreactie lieten zien in vergelijking met de gehanteerde dieren (zowel dexamethason-behandeld als de controles die een injectie kregen met fysiologisch zout). Deze bevindingen versterkten onze hypothese dat het hanteren van dieren tijdens de postnatale periode als een tweede interventiestrategie zou

kunnen dienen, en als zodanig de negatieve bijwerkingen van dexamethason-behandeling zou kunnen tegengaan.

Deze hypothese werd vervolgens getest in **hoofdstuk 5**. We onderzochten de interactie tussen dexamethason-behandeling en het hanteren door dieren met en zonder dexamethason-behandeling al dan niet 3 weken lang dagelijks te hanteren. We zagen dat het hanteren resulteerde in een toename in moedergedrag wanneer de dieren na het hanteren werden teruggeplaatst bij de moeder. Het effect van dexamethason-behandeling op moedergedrag kon in dit design niet worden bestudeerd.

De lange termijn effecten van dexamethason-behandeling werden in grote mate bepaald door het wel of niet hanteren van de dieren. We zagen verschillende typen interacties tussen de effecten van dexamethason en hanteren. Enerzijds werkten de twee effecten in dezelfde richting voor wat de endocriene stressreactie en de startle reactiviteit betrof. Anderzijds werd het aanleren van angstgedrag alleen beïnvloed door het hanteren en niet door dexamethason-behandeling. Daarnaast konden we concluderen dat dexamethason-behandeling de dieren minder ontvankelijk maakte voor de positieve effecten van hanteren op de pre-puls inhibitie en dat niet-gehanteerde dieren gevoeliger zijn voor de effecten van dexamethason-behandeling zoals we zagen bij het ruimtelijk leren. Bovendien zagen we dat de negatieve effecten van dexamethason-behandeling op de ruimtelijke orientatie alleen zichtbaar waren in niet-gehanteerde dieren. Samenvattend lijkt het erop dat de effecten van dexamethason-behandeling het sterkst zijn in niet-gehanteerde dieren, met name in taken met een cognitieve component. Deze bevindingen benadrukken dat de uitkomst van dexamethason-behandeling niet eenduidig is en sterk beïnvloed wordt door andere omgevingsfactoren tijdens het vroege leven.

In **hoofdstuk 6** werden de bevindingen, beschreven in de verschillende hoofdstukken van dit proefschrift samengevat en de implicaties voor het veld van 'developmental programming' werden bediscussieerd. We concludeerden dat - in tegenstelling tot onze verwachting dat het effect van dexamethason-behandeling sterker zou zijn dan dat van andere omgevingsfactoren - de uitkomst van dexamethason-behandeling juist sterk afhankelijk is van andere omgevingsfactoren, en dat deze dus gevoelig is voor interventiestrategieën. Deze bevindingen hebben mogelijk klinische implicaties en benadrukken dat aandacht moet worden besteed aan de invloed van omgevingsfactoren, zoals contact tussen (couveuse) prematuren en de ouders, op het effect van vroege dexamethason-behandeling.

Daarnaast concludeerden we dat zeer subtiele verschillen in moedergedrag invloed hebben op de stressgevoeligheid later in het leven. Deze bevinding benadrukt dat alle omgevingsfactoren tijdens het vroege leven, hoe subtiel ook, bijdragen aan de ontwikkeling van het latere fenotype.

DANKWOORD

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Na vijf jaar is het dan zover: mijn proefschrift is klaar!

Uiteraard wil ik een aantal mensen bedanken die dit proefschrift mede mogelijk hebben gemaakt of de kwaliteit van leven tijdens het proces aanzienlijk hebben verhoogd.

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CURRICULUM VITAE

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Sanne Claessens was born April 23, 1983 in Venray, The Netherlands. In 2001 she passed the Gymnasium exam at Raayland College in Venray and started studying Psychology at Utrecht University. After receiving a BSc degree in Neuropsychology in 2004, she started the MSc programme Neuroscience and Cognition at Utrecht University. She was involved in research projects at the department of Psychonomics under supervision of prof. dr. Edward de Haan and dr. Martine van Zandvoort investigating hemispheric differences in the processing of object colour, and at the department of Psychopharmacology where she studied olfactory bulbectomy related behavioural changes and the effects of psychopharmacology, under supervision of prof. dr. Berend Olivier en dr. Megan Breuer. Her MSc thesis was written under supervision of dr. Mechiel Korte on the topic of CRF related peptides, their receptors and their role in regulating stress responses. The research described in this thesis was initiated in 2007 at the division of Medical Pharmacology of the Leiden/Amsterdam Center for Drug Research (LACDR) and Leiden University Medical Center (LUMC) under supervision of prof. dr. Ron de Kloet and prof. dr. Melly Oitzl. The studies performed during this PhD project were part of the EU funded LifeSpan Network of Excellence. Sanne is currently employed as a medical journalist at Van Zuiden Communications.

LIST OF PUBLICATIONS

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Claessens SE, Belanoff JK, Lucassen PJ, Champagne DL, de Kloet ER. Acute central effects of neonatal dexamethasone treatment: Towards a rescue strategy.
Submitted

Claessens SE, Oitzl MS, Daskalakis NP, de Kloet ER. Developmental and long-lasting consequences of neonatal dexamethasone treatment: Impact of early handling.
Submitted

Claessens SE, Daskalakis NP, Oitzl MS, de Kloet ER. Early handling modulates outcome of neonatal glucocorticoid exposure.
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