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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