Early life experience : neuroendocrine adaptations to maternal absence

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Differential development of stress system (re)activity at weaning dependent on time of disruption of maternal care

Chapter 5

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5.1 Abstract

Maternal deprivation, a separation of mother and pups for 24 hours in the first weeks of life, has long-lasting consequences for the glucocorticoid stress system. These effects are known to depend on the age at deprivation in rats, but were not studied in mice yet. At postnatal day (pnd) 28 (weaning) we measured several markers of the stress system in the hippocampus, hypothalamus and blood plasma. We found, that maternal deprivation is only effective when applied during the SHRP; *i.e.* within the stress hypo-responsive period (SHRP) from pnd 1 to 12. Maternal deprivation early in the SHRP (pnd 3) prolonged the corticosterone response to stress and reduced basal hippocampal GR mRNA expression. Maternal deprivation late in the SHRP (pnd 8) enhanced the amplitude of the ACTH response to stress. A double deprivation (pnds 3 and 8) resulted in sustained, non-responsive high plasma ACTH concentrations with corticosterone levels indistinguishable from control animals. Expression of hippocampal MR and GR mRNA was then decreased. These results underline the impact of the day of postnatal maternal deprivation for the organisation of the stress system in adolescence. Strikingly, a double deprivation did not result in additive effects, but gave an unpredicted neuroendocrine response pattern. We thus conclude, that the developmental stage of the organism determines the vulnerability for the detrimental effects of maternal deprivation.

5.2 Introduction

An undisturbed development of the brain and in particular of systems involved in stress regulation and adaptation, is essential for normal functioning of an organism during adulthood. In humans traumatic early life stress, such as parental separation, childhood sexual or physical abuse, or preterm birth, has been associated with mood and anxiety disorders [6, 7, 10], specifically with (juvenile) onset of major depressive disorder [11, 12]. Adult patients suffering from major depressive disorder, who had experienced early life stress, show persistent hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis and of the autonomous nervous system, as well as an increased sensitivity of these systems to stress [1, 8, 9].

In rodents, a disturbance of normal development during the so-called stress hypo-responsive period (SHRP) has been shown to alter both endocrine and behavioural functions. The SHRP from postnatal day (pnd) 1-12 in mice [2, 3, 33, 35] is characterised by low basal corticosterone concentrations and an inability of mild stressors to induce an endocrine response (pnd 4-14 in rats [17, 28, 31, 32]). Separating mother and pups for 24 hours during the SHRP, *i.e.* maternal deprivation, activates the HPA axis in both rats and mice [2, 18, 27, 35, 36, 40] and, as studied in rats, also leads to a number of long-term changes in HPA axis activity and reactivity to stress [37 - 39, 41].

Separation of mother and pups in rats in various different paradigms or at different ages during the SHRP differentially affects HPA axis responsiveness [15, 16, 23, 26, 39, 40]. Van

Oers *et al.* [39] already called these effects "paradoxical", but they most probably depend on the developmental stage at the time of deprivation. We recently demonstrated in the mouse that the low peripheral activity at normal HPA axis functioning during the SHRP was accompanied by a high level of dynamic changes in mRNA expression profiles of several central components of the HPA axis [33].

Considering these dynamic changes during normal HPA axis development [33], we hypothesise that the age of the pups at separation from the dam strongly affects the long-term effects of maternal deprivation [39]. In the present study we investigated the consequences of the lack of maternal care at different ages during the SHRP for stress system (re)activity in CD1 mice at weaning. We used a single 24 hours maternal deprivation episode early (pnd 3-4) and late (pnd 8) during the SHRP and an episode just outside the SHRP (pnd 13-14). To assess whether repeated maternal deprivations will result in even more severe effects we also deprived mice twice for 24 hours at pnds 3 and 8. At weaning (pnd 28), we tested the (re)activity of the HPA axis by subjecting each mouse to a novel cage. Thereafter, we measured several HPA axis parameters at different time points. The presented data are in agreement with our hypothesis and underline the importance of the pup's age within the mouse SHRP when subjected to maternal deprivation.

5.3 Materials and Methods

5.3.1 Animals

Offspring of CD1 mice (obtained from Charles River, The Netherlands) was used. Four females were mated with one male in type 3 polycarbonate cages containing sawdust bedding and tissue to provide nest-building material; food (SRM-A, Hope Farms, The Netherlands) and water (containing 6% HCl) *ad libitum*; lights on from 7:00 to 19:00 hours in a temperature (21 ± 1°C) and humidity (55 ± 5%) controlled room. Pregnant females were individually transferred to new type 3 polycarbonate cages during the last week of gestation. These females were controlled for litters daily between 9:00 and 9:30 hours. If litters were found, the day of birth was defined as postnatal day 0 (= pnd 0). On the day after parturition, pnd 1, litters were culled to 4 males and 4 females.

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.

5.3.2 Experimental design

Mothers and pups were separated at three different ages during postnatal development; once at pnd 3, 8 or 13 (early and late in and just outside the SHRP, respectively [33]). Also, a combination of pnd 3 and pnd 8 deprivations was used (pnd 3&8).

Mothers nursing litters selected for maternal deprivation were removed from their litters and placed in clean type 3 polycarbonate cages. The home cage containing the pups was placed in

an adjacent room with similar climate conditions on a heating pad (30 – 33°C) to control for pup body temperature. After 24 hours mothers were reunited with their pups and left undisturbed until weaning (pnd 28). Control litters were left undisturbed.

At weaning, pups were tested for their stress responsiveness by solitary exposure to a novel cage. Testing took place between 8:30 and 14:00 hours. The mother was removed from the home cage. One male and one female pup were taken from the nest and sacrificed immediately by decapitation providing a basal sample (0 minutes). The remaining six mice (three males and three females) were then individually placed in a clean type 1 polycarbonate cage with sawdust bedding. After 10, 30 or 120 minutes in this novel environment, two mice (always one male and one female) were sacrificed. Trunk blood from all mice was collected and brains were removed. For each treatment (4 deprivation and 1 control group) and novelty exposure (4 time points) 8 animals per sex were used.

5.3.3 Hormone analysis

Blood plasma was collected individually in potassium-EDTA coated 10 ml tubes (1.6 mg EDTA/ml blood; Sarstedt, Germany). All samples were kept on ice and later centrifuged at 13000 rpm for 15 minutes at 4°C. Blood plasma was transferred to Eppendorf tubes for corticosterone and ACTH determination separately and stored at –20°C until further analysis.

Plasma corticosterone and ACTH levels were measured using a commercially available radio immunoassay (RIA) kit containing ^{125}Iodine labelled corticosterone or ACTH, respectively (ICN Biomedicals Inc., CA, USA). Vials for both RIAs were counted for 2 minutes in a gamma-scintillation counter (Packard Minaxi Gamma counter, Series 5000).

5.3.4 In situ *hybridisation*

Brains were snap frozen in liquid isopenthane on dry ice and stored at –80°C. Animals from the basal novelty condition were used for *in situ* hybridisation. Frozen brains were sectioned at -20°C in a cryostat microtome at 16 µm in the coronal plane through the level of the hypothalamic paraventricular nucleus (PVN) and dorsal hippocampus. Sections were thaw-mounted on poly-L-lysine coated slides (0.001%), air dried and kept at –80°C.

In situ hybridisations using ^{35}S labelled ribonucleotide probes (cRNA probes contained full length coding regions of rat CRH and mouse GR and MR) were performed with some adaptation to the protocol described previously [20]. Briefly, sections were fixed in 4% paraformaldehyde/0.5% glutaraldehyde and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine/HCl. Subsequently, brain sections were dehydrated in increasing concentrations of ethanol. Tissue sections (2 or 4 per slide) were saturated with 100 µl hybridisation buffer (20 mM Tris-HCl (pH 7.4), 50% formamide, 300 mM NaCl, 1 mM EDTA (pH 8.0), 1× Deinhardt's, 250 µg/ml yeast transfer RNA, 250 µl/ml total RNA, 10 mg/ml salmon sperm DNA, 10% dextran sulfate, 100 mM dithiothreitol, 0.1% SDS and 0.1% sodium thiosulfate) containing approximately 1.5 x 10^6 cpm ^{35}S labelled riboprobe. Brain sections were cover slipped and incubated overnight at 55°C.

The following day, sections were rinsed in 2× SSC, treated with RNase A (20 mg/l) and washed in increasingly stringent SSC solutions at room temperature. Finally, sections were washed in 0.1× SSC for 30 minutes at 65°C and dehydrated through increasing concentrations of ethanol. Slides were opposed to Kodak Biomax MR films (Eastman Kodak Co., Rochester, NY) and developed.

Autoradiographs were digitised and relative levels of mRNA expression were determined by computer-assisted optical densitometry (analySIS 3.1, Soft Imaging System GmbH). The average density of 4 - 8 measurements was taken for each animal.

5.3.5 Statistical analysis

Data were analysed by analysis of variance with TREATMENT (age at deprivation) and TIME (exposure times to a novel environment) as fixed factors and the level of significance was set at $P<0.05$. When appropriate this was followed by Tukey's or LSD *post hoc* comparisons. All data are presented as mean ± S.E.M.

5.4 Results

All mice showed a stress response for corticosterone and ACTH. Though sex effects were observed, in further analyses and discussion of both peripheral and central markers of the HPA axis only males are presented. Female mice did not show a treatment effect on the endocrine response (data not shown).

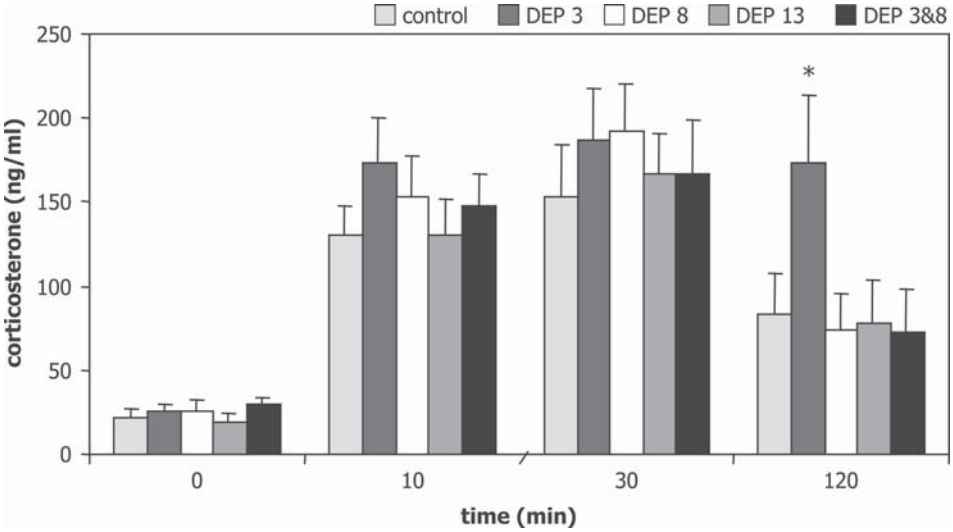

Figure 5.1
Plasma corticosterone concentration (ng/ml) at 28 days of age: basal (t=0 minutes) and at 10, 30 and 120 minutes after exposure to a clean novel cage. Mouse pups had been deprived from their mother for 24 hours once at either pnd 3 (DEP 3), 8 (DEP 8) or 13 (DEP 13); or in a combination of pnd 3 and 8 deprivations (DEP 3&8). Control mice remained undisturbed throughout their development. Data represent mean ± S.E.M., * $P<0.05$ (significant from all other groups at t=120 minutes).

5.4.1 Corticosterone (*Figure 5.1*)

We observed a main effect of time (F(3,159)40.10, *P*<0.001); main treatment effected passed statistical significance (*P*=0.07). Under basal conditions all groups showed similar low corticosterone concentrations. Novelty induced a stress response in all groups, demonstrated by elevated corticosterone levels at 10 and 30 minutes. After 120 minutes, pnd 3 deprived animals still showed significantly elevated corticosterone, whereas corticosterone of the other groups had returned to basal levels (*P*<0.05 versus all other groups). Interestingly, an extra deprivation at pnd 8 abolished the effects induced by maternal deprivation at pnd 3.

5.4.2 ACTH (*Figure 5.2*)

We observed a main effect of time (ANOVA: F(3,159)21.56, *P*<0.001), but not for treatment (*P*=0.61). The interaction between time and treatment (F(12,159)1.87, *P*<0.05) indicated that the treatment groups showed different time courses. Besides pnds 3&8 deprived animals all groups were able to elicit a stress response upon placement in a novel environment. Under basal conditions pnds 3&8 deprived animals had already significantly higher ACTH concentrations (*P*<0.05) than all other groups and did not respond to novelty. Furthermore, pnd 8 derpived animals showed the highest ACTH concentrations 30 minutes after novelty (*P*<0.05 versus control).

5.4.3 In situ *hybridisations* (*Table 5.1*)

CRH mRNA expression in the amygdala and PVN showed no main effect of treatment (amygdala: F(4,32)0.20, *P*=0.94; PVN: F(1,32)1.89, *P*=0.14) and GR mRNA expression in the PVN (F(1,34)0.94, *P*=0.45) remained unchanged as well. In parvo- and magnocellular neurons of the PVN AVP mRNA expression was not affected by treatment (parvocellular neurons: F(4,32)1.24, *P*=0.32; magnocellular neurons F(4,32)0.78, *P*=0.55).

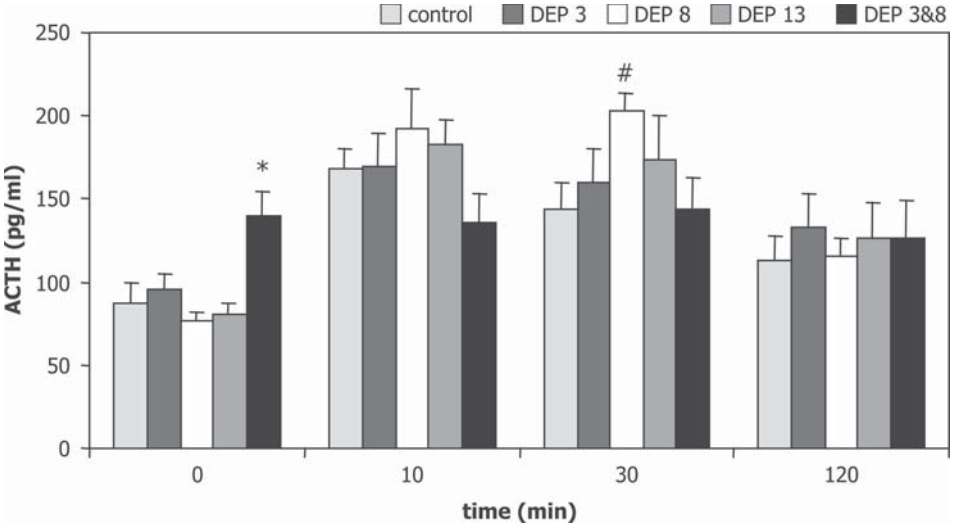

Figure 5.2
Plasma ACTH concentration (pg/ml) in mouse pups at 28 days of age: basal (t=0 minutes) and at 10, 30 and 120 minutes after exposure to a clean novel cage. Mouse pups were deprived from their mother for 24 hours once at either pnd 3 (DEP 3), 8 (DEP 8) or 13 (DEP 13); or in a combination of pnd 3 and 8 deprivations (DEP 3&8). Control mice remained undisturbed throughout their development. Data represent mean ± S.E.M., * *P*<0.05 (significant from all other groups at t=0 minutes), # *P*<0.05 (significant from control mice a t=30 minutes).

Table 5.1: Body weight and mRNA expression for central HPA axis markers

		control	DEP 3	DEP 8	DEP 13	DEP 3&8
body weight		21.38 ±0.52	**20.88** ±0.33[b]	21.44 ±0.21	21.91 ±0.23	**20.45** ±0.31[a]
amygdala	CRH	30.10 ±1.80	29.43 ±2.74	31.93 ±2.97	29.19 ±2.47	29.15 ±2.84
PVN	CRH	44.90 ±3.32	40.79 ±3.24	32.88 ±3.02	47.15 ±4.49	39.50 ±2.07
	pAVP	335.85 ±36.74	284.15 ±31.68	388.31 ±31.92	360.94 ±39.83	314.37 ±34.15
	mAVP	393.75 ±38.95	328.08 ±40.06	427.84 ±50.18	403.37 ±45.31	371.21 ±45.89
	GR	60.85 ±4.04	59.69 ±2.58	55.65 ±3.21	55.54 ±1.89	53.32 ±4.04
hipp.	MR	62.76 ±2.46	58.92 ±2.30	55.90 ±4.47	55.50 ±5.03	**49.94** ±2.06*
	GR	41.13 ±1.09	**36.04** ±2.18*	37.04 ±2.63	37.14 ±1.32	**29.50** ±0.72[#]

Mouse pups were deprived from their mother for 24 hours, either once at pnd 3 (DEP 3), 8 (DEP 8) or 13 (DEP 13); or in a combination of pnd 3 and 8 deprivations (DEP 3&8) and tested at 28 days of age (weaning). Control mice remained undisturbed throughout their development. Body weight is expressed in grams (g) and was measured after decapitation (head + body). Relative levels of mRNA expression are expressed in optical density (O.D.). Corticotropin releasing hormone (CRH) expression was measured in the amygdala. CRH, vasopressin (measured in parvo- (pAVP) and magnocellular (mAVP) neurons) and glucocorticoid receptor (GR) expressions were measured in the paraventricular nucleus of the hypothalamus (PVN). Mineralo- and glucocorticoid receptor (MR and GR) expressions were measured in the hippocampus (hipp.). All data are presented as mean ± S.E.M., significant effects are presented in bold, * $P<0.05$ versus "control", [#] $P<0.01$ versus all other groups, [a] $P<0.05$ versus DEP 8 or DEP 13, [b] $P<0.05$ versus DEP 13.

As all hippocampal subregions showed the same pattern of expression of GR or MR mRNA, these data were pooled. For GR mRNA, we observed a main effect of treatment ($F(4.34)4.17$, $P<0.01$) with significantly lower basal expression levels in pnd 3 deprived versus control animals ($P<0.05$) and in pnds 3&8 deprived animals versus all other groups ($P<0.01$).

MR mRNA expression was significantly lower in pnds 3&8 deprived animals than in control animals ($P<0.05$).

5.4.4 Body weight (*Table 5.1*)

Body weight was determined after decapitation at different time points after introduction to a novel environment as a general marker for pup development. There was no effect of time ($F(3,159)2.16$, $P>0.10$), so data were collapsed across this variable. A main effect of treatment was observed ($F(4,159)2.70$, $P<0.05$). Test statistics of pnd 3 and pnd 13 deprived animals compared to controls just passed significance. Deprivation at pnd 3 resulted in significantly lower body weight compared to deprivation at pnd 13 ($P<0.05$). Deprivation at pnds 3&8 resulted in significantly lower body weight compared to deprivation at postnatal day 8 or 13 ($P<0.05$).

5.5 Discussion

Our experiments demonstrated that a single 24 hours maternal deprivation exclusively inside the SHRP altered the HPA axis (re)activity of adolescent CD1 mice, depending on the age at deprivation. Early in the SHRP (pnd 3) maternal deprivation resulted in an augmented corticosterone response, which was accompanied by reduced GR mRNA expression in the hippocampus, whereas a late deprivation (pnd 8) resulted in an ACTH response with an elevated amplitude. Furthermore, a double deprivation (pnd 3&8) interfered with the effects induced by a

previous deprivation resulting in sustained high ACTH concentrations non-responsive to stress, but gave a corticosterone response comparable to control animals.

5.5.1 Deprivation early (pnd 3) and late (pnd 8) in the SHRP

Maternal deprivation of mice early in the SHRP (pnd 3) resulted in a prolonged corticosterone response to novelty at weaning (pnd 28). Pnd 3 deprived mice also had lower hippocampal GR expression, while other HPA axis markers remained unaltered. In contrast, reduced GR expression in the PVN and pituitary were observed for adult rats deprived at pnd 3 [29], while hippocampal expression and binding capacities remained unaltered [23, 29]. Since the MR and GR in the hippocampus are an important feedback site regulating the endocrine stress response [4, 21], the observed reduced expression of GR, responsible for a suppression of the activated HPA axis, might explain the prolonged corticosterone response.

Deprivation late in the SHRP (pnd 8) did not result in an altered corticosterone response, but gave an ACTH response with higher peak levels after 30 minutes of novelty. These data suggest that, as in rats [23, 39, 40, 42], deprivation late in the SHRP results in reduced adrenal sensitivity to ACTH. However, no alteration in expression of HPA axis markers was observed that could explain this enhanced ACTH response. Although parvocellular AVP expression was not altered, AVP might still be able to enhance CRH activity at the level of the pituitary [22] resulting in a higher ACTH release. On the other hand, pituitary sensitivity to CRH and/or AVP might be increased [19].

Interestingly, body weight at weaning of pnd 3 deprived animals was slightly reduced, whereas this was unaffected in animals deprived at pnd 8. In rats, a reduced body weight was observed for both the early and late deprived animals, which was accompanied by a reduced caloric intake in early deprived animals only [23]. Whether the observed reduced body weight in our mice is also caused by altered feeding behaviour remains to be investigated.

Since the HPA axis is at different developmental stages at the time of maternal deprivation [33] the variable long-term consequences might be due to the different direct effects of maternal deprivation. Maternal deprivation at pnd 8 decreased CRH and GR mRNA expression in the PVN and MR and GR mRNA expression in the hippocampus directly after the 24 hours of maternal absence in the same mouse strain [35]. Strikingly, no long-term alterations were observed in the current study, indicating a recovery from these profound immediate effects. On the other hand, directly after a deprivation procedure at pnd 3 reduced CRH and GR mRNA expression in the PVN were observed, whereas hippocampal MR and GR mRNA expression remained unaltered [***Chapter 6***]. At weaning the effects in the PVN were abolished, whereas hippocampal GR mRNA expression was lower compared to control animals, indicating that maternal deprivation did affect further development. Overall, however, our data indicate that mice are able to recover from most of the immediate effects of maternal deprivation and investigations of the altered HPA axis development are necessary to understand the cause of long-term effects in more detail.

In rats tested at 2-3 months of age also a prolonged corticosterone response has been reported for a deprivation early in the rat SHRP (pnd 5) [23]. However, at weaning (pnd 20 in rats) others reported that maternal deprivation at pnd 3 resulted in an exaggerated ACTH response [39], whereas in 5 and 20 months old rats no effect of early maternal deprivation (pnd 4) was observed [16]. In accordance with our data of a late deprivation, a late deprivation in rats (pnd 14) has been shown to result in an exaggerated ACTH response at 2-3 months of age [23], but an attenuated ACTH response due to maternal deprivation at pnd 11 is reported at weaning [39]. At 5 and 20 months of age no effects were observed of deprivation at pnd 9 [16].

Furthermore, since the onset and duration of the SHRP as determined by endocrine responses already differs between mice and rats [17, 32, 33, 42], central markers of the HPA axis will probably also have distinct developmental patterns [33], complicating a comparison between both species. This is substantiated by the direct effects of maternal deprivation on HPA axis markers, of which most, but not all, are the same in mice and rats (previously discussed by Schmidt *et al.* [35]). These results indicate that the exact day at which the maternal deprivation is performed as well as the age and the species in which the consequences are tested are of eminent importance [14, 23].

5.5.2 Deprivation outside (pnd 13) the SHRP

Maternal deprivation outside the SHRP did not affect basal or stress-induced ACTH and corticosterone or expression of central HPA axis markers at weaning. This is in contrast to observations in rats that indicated that maternal deprivation at pnd 18, well outside the rat SHRP, was able to increase stress-induced corticosterone at 5 months of age [16]. Furthermore, "early" weaning at pnd 20, thus also well outside the rat SHRP, did immediately affect the HPA axis at multiple levels, resulting in elevated basal and stress-induced corticosterone at pnd 21 and reduced ACTH, paraventricular *c-fos* and CRH mRNA expression and hippocampal GR mRNA expression [5, 35]. Direct effects are very plausible in our pnd 13 deprived mice as well, since the HPA axis at this age does not show full adult characteristics yet [33]. These data indicate that long-term consequences of maternal deprivation in mice may, in contrast to rats, only be achieved by modulating the HPA axis development within the SHRP.

5.5.3 Repeated deprivation (pnds 3&8)

In order to induce a more robust disturbance of the HPA axis, we applied a combination of pnd 3 and pnd 8 deprivations. Interestingly, this extra deprivation at pnd 8 completely abolished the prolonged corticosterone response induced by deprivation at pnd 3. Intriguingly, these double deprived animals did show increased basal ACTH levels and an inability to respond to a novel environment with an increase in ACTH.

Basal expression of MR mRNA in the hippocampus was lower compared to controls. The expression of GR mRNA was even lower than the decreased expression caused by deprivation at pnd 3 and indicated that more deprivation events resulted in larger effects. Since both MR and

GR decreased it remains questionable whether these alterations also lead to an altered balance in MR and GR protein levels and whether this altered balance is able to (partly) explain the observed endocrine effects.

As indicated before [23], a single 24 hours deprivation model has an advantage over repeated separation models, since it enables the dissociation of effects taking place at different developmental ages. However, in studies of long-term consequences of traumatic early life events in rodents, the use of repeated maternal separations is a more often used paradigm [13, 24, 25, 30]. The direct effects on HPA axis development have not yet been investigated thoroughly, but our studies clearly substantiate other data indicating that the changes of the HPA axis by maternal deprivation depend on the developmental age at which the procedure is started and ended [23, 33, 39] and by the duration of separation [34].

5.5.4 Conclusions

We observed that a single maternal deprivation produced long-term changes in the HPA axis, both at the basal set point and in responsiveness to a mild stressor, but only when applied within the SHRP. Our data indicate that mice are able to recover from most of the immediate effects induced by a single deprivation episode. These data further substantiate other studies indicating that the consequences of maternal deprivation depend on the age at which this procedure is applied, the age at which the consequences are determined, the species used and that subsequent deprivations interfere with the effects induced by an earlier deprivation.

5.6 Acknowledgements

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