
Universiteit
Leiden
The Netherlands

Early life experience : neuroendocrine adaptations to maternal absence

Enthoven, L.

Citation
Enthoven, L. (2007, October 4). *Early life experience : neuroendocrine adaptations to maternal absence*. Retrieved from https://hdl.handle.net/1887/12379

Version:	Corrected Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/12379

Note: To cite this publication please use the final published version (if applicable).

Ontogeny of the HPA axis of the CD1 mouse following 24 hours of maternal deprivation at pnd 3

Chapter 6

L. Enthoven, M.V. Schmidt, Y.H. Cheung,
M. van der Mark, E.R. de Kloet & M.S. Oitzl

6.1 Abstract

Maternal deprivation during the stress-hypo-responsive period (SHRP) has short- and long-term consequences for the hypothalamic-pituitary-adrenal (HPA) axis in mice and rats. Recovery or persistence of the neuroendocrine effects on the days following the trauma is the focus of this study. Mouse pups were deprived from their mother on postnatal day (pnd) 3 for 24 hours, resulting in elevated basal and stress-induced corticosterone and reduced CRH mRNA expression in the paraventricular nucleus (PVN) of the hypothalamus. Reunion with the mother on pnd 4 suppressed basal and stress-induced corticosterone below levels of non-deprived control pups for at least three days. ACTH was lower on pnd 5, CRH mRNA expression in the PVN was suppressed for two days, but exceeded control levels at pnd 7 to follow the slow decline of CRH mRNA in controls until pnd 12. GR mRNA expression in the hippocampal CA1 area showed a developmental arrest with a delayed increase over the following days. Hippocampal MR mRNA expression was not affected. From pnd 9 onwards, maternally deprived and control mice showed a similar response pattern of HPA axis markers emerging from the SHRP. HPA axis markers responded with an unexpected dynamic pattern to restore homeostasis. Since the immediate effects of maternal deprivation are partially age-dependent, we conclude that the stage of brain development will determine the susceptibility and the regeneration capacity of the various brain systems to environmental disturbances.

6.2 Introduction

One of the main characteristics of the developing neuroendocrine stress system in rats and mice is the so-called stress hypo-responsive period (SHRP). Lasting from about postnatal day (pnd) 1 to 12 in mice [6, 20] and pnd 4 to 14 in rats [12, 18, 19, 36], this period is mainly characterised by low levels of corticosterone and a reduced corticosterone secretion in response to mild stressors. Separating mother and pups for 24 hours (maternal deprivation) during this period results in an activation of the hypothalamic-pituitary-adrenal (HPA) axis, expressed by elevated corticosterone immediately after deprivation, together with an increased responsiveness of the HPA axis to mild stress [6, 12, 17, 23, 29].

Maternal deprivation serves as an animal model to study the consequences of traumatic early life events, which are considered risk factors for the development of mood disorders in humans [5, 15]. Studies in rats have shown that maternal deprivation, as well as the application of corticosteroids initiate a number of long-term changes in neuroendocrine and behavioural systems [2, 9, 16, 27, 35]. Complex relationships with the age and duration of deprivation, as well as on gender and strain have been reported [27 - 29, 35, 36]. There is a lack of data on stress system (re)activity on the days subsequent to maternal deprivation that might help to understand the long-term effects. Levine and colleagues demonstrated that once stress responsivity has been induced by maternal deprivation, reunion with a lactating female results in suppression of a rat

pup's corticosterone response to novelty [17, 26], indicating the normalisation of the hormonal response to the stress hypo-responsive state. Furthermore, the short, but daily separations of mother and pups (±15 minutes from birth until weaning, *i.e.* handling) increased maternal care and accelerated the maturation of an adult-like circadian corticosterone rhythm [1]. To better understand the long-term effects of maternal deprivation it is essential to know how the maternal deprivation-induced HPA axis' (re)activity progresses on the days after the deprivation, *i.e.* upon reunion with the mother. Will the immediate effects of maternal deprivation prevail, be compensated or alleviated and what will be the dynamics of these responses? Will maternal deprivation influence the duration of the SHRP?

We hypothesised that a single 24 hours maternal deprivation will alter the subsequent developmental pattern of HPA axis (re)activity markers. Moreover, we expected that maternal deprivation would not only disrupt, but also change the duration of the SHRP. In previous experiments, we had deprived mice in the late phase of the SHRP (pnd 8 to 9) [23, 24]. Maternal deprivation at an earlier stage of the SHRP will allow us to follow the development of the HPA system during the SHRP more accurately within a longer time frame. Thus, we maternally deprived CD1 mouse pups from pnd 3 to 4 and reunited them with their mother. We measured the several markers of HPA axis (re)activity (plasma ACTH and corticosterone, hippocampal MR and GR mRNA expression, expression of GR and CRH in the PVN) on the subsequent days (pnd 4-13) in two separate experiments.

6.3 Materials and Methods

6.3.1 Animals

Offspring of CD1 mice (obtained from Charles River, The Netherlands) was used in this study. After a habituation period of two weeks, three females were mated with one male in type 3 polycarbonate cages (820 cm^3) 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 clean 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) for that litter. On the day after parturition, pnd 1, litters were culled to eight healthy pups (four males and four females).

All 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. The protocols were approved by the Animal Care Committee of the Faculty of Medicine, Leiden University (Leiden, The Netherlands).

6.3.2 Experimental design

We studied the development of central and peripheral changes of HPA axis markers following a 24 hours maternal deprivation at pnd 3: (Experiment I) to asses whether changes induced by maternal deprivation remained after reunion with the mother and (Experiment II) to asses whether maternal deprivation influenced the duration of the SHRP.

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 on a heating pad (30-33°C) to control for pup body temperature under similar climate conditions as mentioned above. Food and water were not available for the pups during this period. Except for litters tested at pnd 4, mothers were reunited with their pups after 24 hours and left undisturbed until testing. Control litters were left undisturbed.

Testing took place between 9:00 and 12:00 hours. On test days, four pups per nest (two males and two females) were sacrificed immediately providing a basal sample. The remaining pups were individually placed in a clean novel cage on a heating pad to induce novelty stress and sacrificed 30 minutes later. Every treatment group consisted of two litters (n=8). Animals were sacrificed by decapitation and trunk blood was collected individually in labeled 1.5 ml EDTA-coated microcentrifuge tubes. All blood samples were kept on ice and later centrifuged for 15 minutes at 13000 rpm at 4°C. Plasma was transferred to clean, labeled 1.5 ml Eppendorf tubes. All plasma samples were stored frozen at -20°C until determination of corticosterone and ACTH concentrations. Whole heads (without skin and jaws) were removed, snap frozen in isopentane on dry ice and stored at –80°C for *in situ* hybridisation.

6.3.3 Hormone 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). Corticosterone concentrations were determined in duplicate from an extended standard curve (0, 12.5, 25, 50, 100, 250, 500, 1000 ng corticosterone/ml). Vials for either RIA were counted for 2 minutes in a gamma-scintillation counter (Packard Minaxi Gamma counter, Series 5000).

6.3.4 In situ *hybridisation*

Deprived and control animals from the basal condition only 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 0.01% poly-L-lysine coated slides, air dried and kept at -80°C.

In situ hybridisations using ^{35}Sulphur labelled ribonucleotide probes for corticotrophin releasing hormone (CRH), glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) were, with some adaptations, performed as described previously [14]. 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 alcohol. The probes contained the full length coding regions of CRH (rat), GR and MR (mouse). The antisense probes were transcribed from a linearised plasmid. Tissue sections were saturated with 100 µl hybridisation buffer containing 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 fish sperm DNA, 10% dextran sulfate, 100 mM dithiothreitol, 0.1% SDS, 0.1% sodium thiosulfate and supplemented with approximately 1.5 x 10^6 cpm ^{35}Sulphur labelled riboprobe. Brain sections were cover slipped and incubated overnight at 55°C. The next 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 alcohol. Slides were opposed to Kodak Biomax MR film (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 measurements was taken for each animal.

6.3.5 Statistical analysis

Data were analysed by analysis of variance (ANOVA) with TREATMENT (control or deprivation), AGE (for experiment I: pnds 4, 5, 6 or 7; for experiment II: pnds 4, 7, 8, 9, 10, 11, 12 and 13) and TIME (exposure to novel environment for 0 or 30 minutes) as fixed factors. The level of significance was set at $P<0.05$. When appropriate this was followed by Tukey's *post hoc* comparisons. After determination that there were no differences between sexes, data were collapsed across this variable. All data are presented as mean ± S.E.M.

6.4 Results

6.4.1 <u>Experiment I</u>: To asses whether changes induced by maternal deprivation will remain after reunion with the mother, pups were tested for their HPA axis (re)activity at pnds 4, 5, 6 and 7.

6.4.1.1 Corticosterone (***Figure 6.1***)

Maternal deprivation affected corticosterone secretion dependent on the age of testing (interaction treatment x age: $F(3,159)43.48$, $P<0.001$). At pnd 4 immediately after maternal deprivation we observed elevated basal corticosterone concentrations ($P<0.05$), though 30 minutes of exposure to a novel environment did not further increase the circulating corticosterone level. At pnd 5 basal corticosterone concentrations were lower than those observed for control animals ($P<0.05$) and pups showed no corticosterone response to novelty. Moreover, deprived animals did not recover from this suppression of (re)activity during the following two days (pnds 6 and 7). Control animals had low basal corticosterone levels and even showed a slight, but statistically significant increase in response to novelty. However, the maternal deprivation induced corticosterone increase at pnd

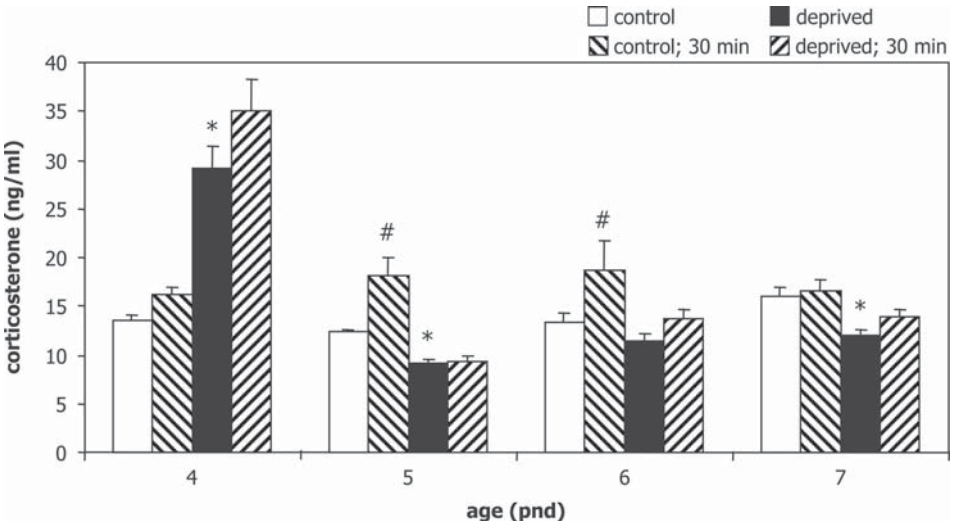


Figure 6.1
Basal and stress-induced plasma corticosterone levels in control and deprived mouse pups at four postnatal days after maternal deprivation. Data represent mean ± S.E.M., * $P<0.05$ significant from control animals at the same day and treatment group, # $P<0.05$ significant from basal animals within the same treatment group.

4 exceeded the novelty-induced increase in the control group.

6.4.1.2 ACTH

Maternal deprivation influenced ACTH release (treatment: $F(1,144)8.59$, $P<0.01$). *Post hoc* analyses revealed that at pnd 5 basal ACTH concentrations of the deprived group were below control values (ACTH in pg/ml; control 28.74 ±1.07; deprived 22.52 ±1.00). Basal ACTH concentrations at pnds 4, 6 and 7 and stress-induced ACTH concentrations at pnds 4, 5, 6 and 7 were not affected by maternal deprivation. Control animals did not show ACTH responses to novelty.

6.4.1.3 CRH mRNA expression in PVN (***Figure 6.2***)

Maternal deprivation significantly affected CRH mRNA expression in the PVN (treatment: $F(1,73)29.23$, $P<0.001$), dependent on the age of testing (interaction treatment x age: $F(3,73)25.62$, $P<0.001$). *Post hoc* analysis revealed that after maternal deprivation CRH expression was reduced by more than 50% at pnds 4 and 5, restored to control expression again at pnd 6 and exceeded controls at pnd 7. Throughout these four days, CRH mRNA expression in control animals remained unchanged.

6.4.1.4 GR mRNA expression in PVN and hippocampus (***Figure 6.3***)

GR mRNA expression in the PVN (***A***) was affected by maternal deprivation (treatment: $F(1,73)8.61$, $P<0.01$) and dependent on the age of testing (interaction treatment x age: $F(3,73)6.45$, $P<0.01$). GR mRNA expression was lower in deprived animals on pnd 4; from pnd 5 onwards, GR mRNA expression recovered to the same levels as observed in control animals. Expression in control animals remained constant.

At this age GR mRNA expression can only be measured in the CA1 subfield of the hippocampus. In the CA3 and the dentate gyrus subfields GR mRNA expression is below the

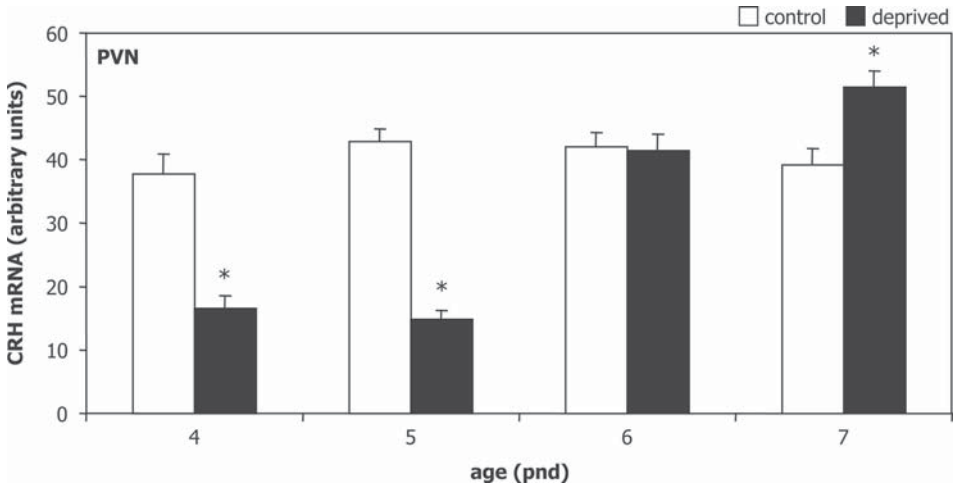
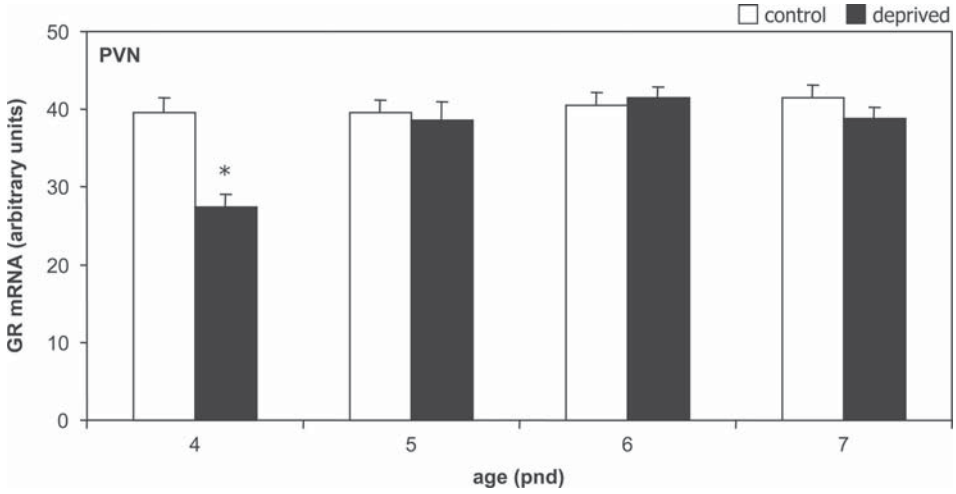


Figure 6.2
Basal expression levels of CRH mRNA in the paraventricular nucleus of the hypothalamus (PVN) for control and deprived mouse pups at four postnatal days after maternal deprivation. Data represent mean ± S.E.M., * $P<0.05$ significant from control animals at the same day.

A



B

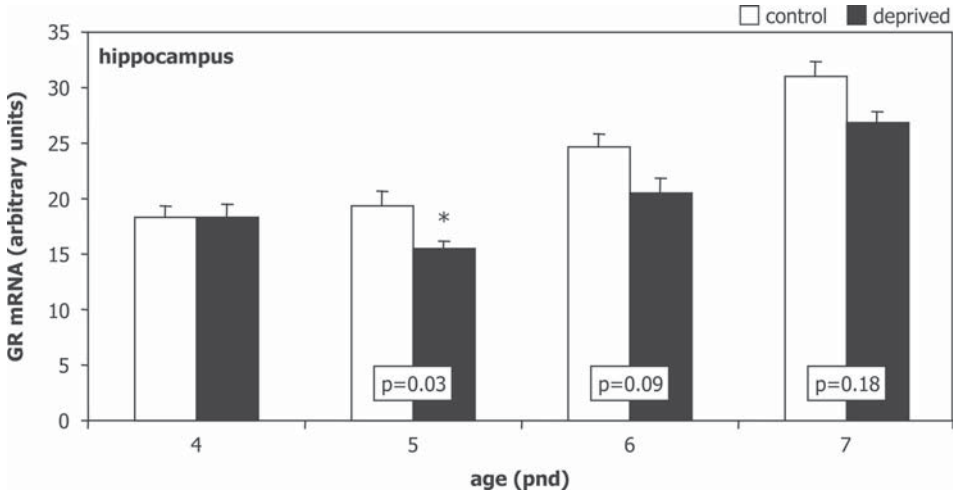


Figure 6.3
Basal expression levels of GR mRNA in the paraventricular nucleus of the hypothalamus (PVN) (***A***) and the CA1 area of the hippocampus (***B***) for control and deprived mouse pups at four postnatal days after maternal deprivation. Data represent mean ± S.E.M., * $P<0.05$ significant from control animals at the same day.

Table 6.1: Basal expression levels of MR mRNA in the CA1, CA2, CA3-4 and dentate gyrus (DG) area of the hippocampus for control and deprived mouse pups at postnatal days (pnd) 4 to 7 after maternal deprivation.

Age (pnd)	4	5	6	7
Control animals:				
CA1	31.0 ±1.9	28.6 ±1.2	29.44 ±1.3	30.0 ±1.6
CA2	88.2 ±2.9	89.4 ±1.3	88.1 ±2.4	88.7 ±3.8
CA3-4	55.4 ±2.2	54.0 ±0.4	53.3 ±2.8	48.7 ±2.0
DG	**57.6** ±2.0	**58.6** ±0.3	**61.6** ±3.2	**67.7** ±2.7
Deprived animals:				
CA1	29.5 ±1.1	31.6 ±0.5	33.4 ±2.1	33.2 ±1.4
CA2	83.8 ±2.6	86.7 ±2.2	87.8 ±2.5	86.5 ±4.1
CA3-4	54.2 ±1.7	53.6 ±2.4	56.7 ±2.2	55.1 ±2.2
DG	**56.7** ±1.8	**59.3** ±4.1	**70.1** ±4.5	**73.5** ±1.9

Data are arbitrary units of mRNA expression and represent mean ± S.E.M. **Bold** indicates a significant increase in expression with age ($P<0.05$).

detection limit. ANOVA revealed that maternal deprivation significantly affected GR expression development in the CA1 area (treatment: $F(1,79)8.17$, $P<0.001$). Control and deprived groups showed a gradual increase in GR mRNA expression with age (age: $F(3,32)13.97$, $P<0.001$) (***B***). *Post hoc* analysis revealed that deprived animals had comparable expression levels immediate after maternal deprivation at pnd 4. On pnd 5, expression was significantly lower in deprived animals ($P=0.03$). This difference in expression gradually decreased during the next two days (pnd 6: $P=0.09$; pnd 7: $P=0.18$).

6.4.1.5 MR mRNA expression in hippocampus (*Table 6.1*)

MR mRNA expression was measured in the CA1, CA2 and CA3-4 areas and the dentate gyrus of the hippocampus. In both control and deprived animals the expression in the CA1, CA2 and CA3-4 area of the hippocampus remained constant throughout these four days of testing and maternal deprivation did not affect the expression of MR mRNA in these areas. MR mRNA expression in the dentate gyrus gradually, but significantly increased with age in both control ($F(3,32)3.52$, $P<0.05$) and deprived animals ($F(3,32)6.96$, $P<0.01$).

6.4.2 Experiment II: To asses whether maternal deprivation influences the duration of the SHRP, pups were tested for their peripheral HPA axis (re)activity at pnds 4, 7, 8, 9, 10, 11, 12 and 13. Pups were also tested for those central HPA axis markers that still showed a treatment effect four days after reunion (experiment I: CRH mRNA in the PVN, GR mRNA in the hippocampus).

6.4.2.1 Corticosterone (*Figure 6.4*)

As expected maternal deprivation affected corticosterone secretion, but this was dependent on the age of testing (interaction treatment x age: $F(7,198)4.69$, $P<0.001$). At pnd 4 maternal deprivation resulted in elevated basal corticosterone concentrations ($P<0.05$) and 30 minutes of novelty further increased the circulating corticosterone levels ($P<0.05$). At pnd 7 basal corticosterone concentrations were comparable to those observed for control animals. At that time there was

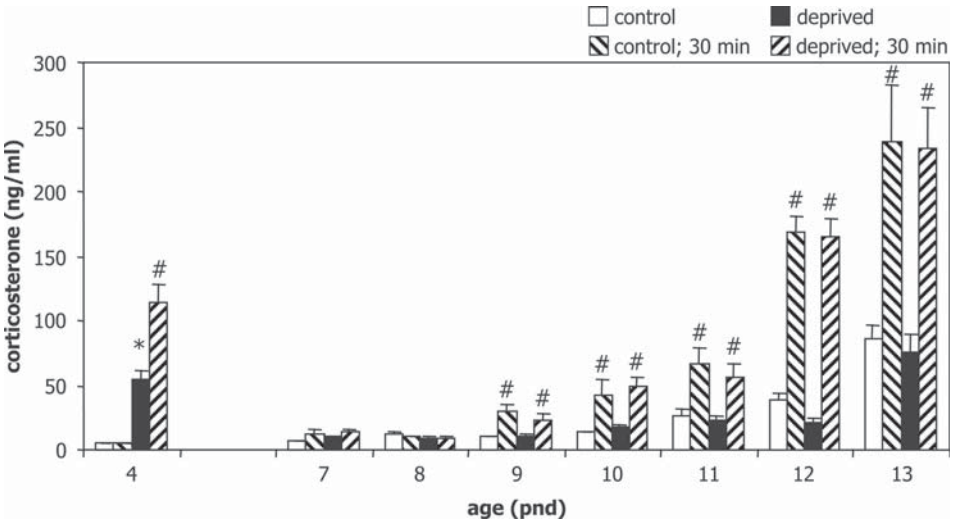


Figure 6.4
Basal and stress-induced plasma corticosterone levels in both control and deprived mouse pups at postnatal days 4 and 7 to 13 after maternal deprivation at pnd 3. Data represent mean ± S.E.M., * $P<0.05$ significant from control animals at the same day and treatment group, # $P<0.05$ significant from basal animals within the same treatment group.

no corticosterone response to novelty in either control or deprived animals. The emergence of the HPA axis from the SHRP was only affected by age ($F(7,198)69.88$, $P<0.001$) and time ($F(1,198)129.79$, $P<0.001$) and an interaction between these two factors (age x time: $F(7,198)26.16$, $P<0.001$). Maternal deprivation had no effect. From pnd 9 onwards 30 minutes of isolated novelty exposure significantly increased circulating corticosterone levels ($P<0.05$) in both control and deprived animals. Furthermore, basal and novelty-induced corticosterone levels were comparable between the two treatment groups and gradually increased with age.

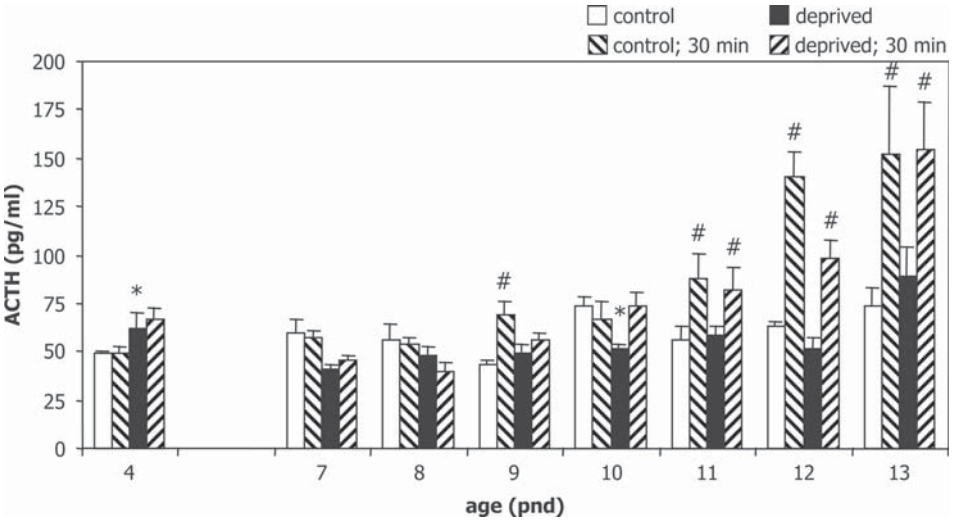


Figure 6.5
Basal and stress-induced plasma ACTH levels in both control and deprived mouse pups at postnatal days 4 and 7 to 13 after maternal deprivation at postnatal day 3. Data represent mean ± S.E.M., * $P<0.05$ significant from control animals at the same day and treatment group, # $P<0.05$ significant from basal animals within the same treatment group.

6.4.2.2 ACTH (*Figure 6.5*)

Similar as observed for corticosterone, ACTH release was not influenced by treatment, but only by age ($F(7,198)17.41$, $P<0.001$), time ($F(1,198)36.71$, $P<0.001$) and an interaction between these two factors (interaction age x time: $F(7,198)6.98$, $P<0.001$). *Post hoc* analyses revealed that basal ACTH levels were increased immediately after maternal deprivation at pnd 4 ($P<0.05$). Thirty minutes novelty did not further increase these levels. At pnds 7 and 8 basal and stress-induced ACTH was comparable between control and deprived animals. At pnd 9 a novel environment significantly increased ACTH in control animals only ($P<0.05$). For deprived animals basal levels were significantly lower compared to controls at pnd 10 ($P<0.05$) and novelty was able to induce a response ($P<0.05$). From pnd 11 onwards 30 minutes of isolated novelty significantly increased circulating ACTH concentrations ($P<0.05$) in both control and deprived animals. Furthermore, novelty-induced ACTH concentrations gradually increased with age in either treatment group.

6.4.2.3 CRH mRNA expression in PVN (*Figure 6.6*)

Maternal deprivation affected CRH mRNA expression in the PVN, dependent on the age of testing (interaction treatment x age: $F(7,102)10.42$, $P<0.001$). At pnd 4 CRH expression was significantly reduced by more than 50% ($P<0.01$), whereas it was increased by about 25% at pnds 7 and 8 ($P<0.05$). From pnd 9 onwards, CRH mRNA expression of control animals started to decline. At this same age CRH mRNA expression of deprived animals declined, but at a faster rate, reaching control values at pnd 11. From pnds 11 to 13 expression levels remained comparable between all animals tested.

6.4.2.4 GR mRNA expression in hippocampus

Due to technical problems the *in situ* hybridisations could not be performed successfully and, unfortunately, therefore all sectioned brain material was lost.

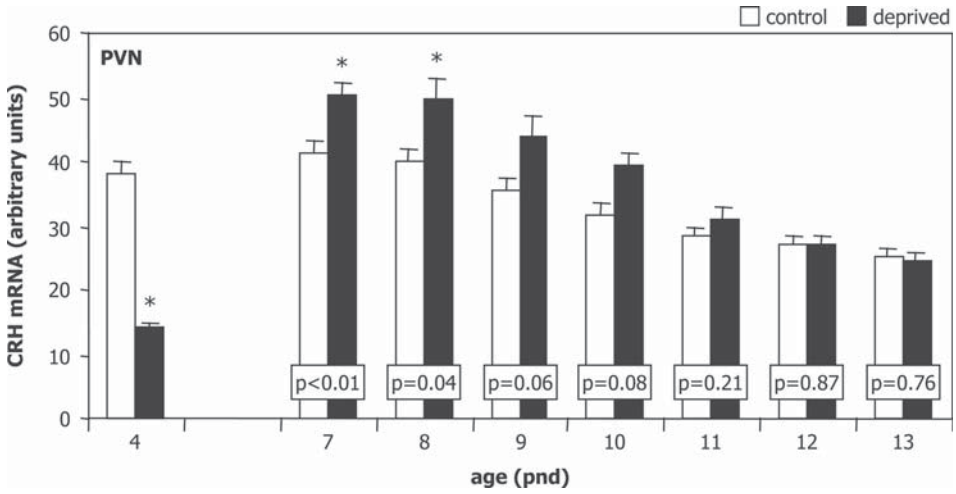


Figure 6.6
Basal expression levels of CRH mRNA in the paraventricular nucleus of the hypothalamus (PVN) for control and deprived mouse pups at postnatal days 4 and 7 to 13 after maternal deprivation at postnatal day 3. Data represent mean ± S.E.M., * $P<0.05$ significant from control animals at the same day.

6.5 Discussion

A single 24 hours maternal deprivation at pnd 3 has direct consequences for and changes the developmental pattern of the activity and reactivity of the glucocorticoid-related stress system. The duration of the stress hypo-responsive period (SHRP) remained comparable to control mice. Deprivation from and reunion with the mother were accompanied by characteristic changes of HPA axis markers with different recovery patterns over time. The direct effects of maternal deprivation are partly in accordance with findings of other maternal deprivation studies in rats and mice, but differ in some aspects. Novel are the data of the developmental patterns of distinct HPA axis activity markers after reunion with the mother.

6.5.1 Direct effects of maternal deprivation

The elevation of basal corticosterone and the increased corticosterone response to novelty directly after deprivation are in line with other maternal deprivation studies in rats and mice [6, 13, 17, 23, 25, 29]. Also the lower expression of CRH and GR mRNA in the PVN corresponds to other data [8, 23, 24, 33]. In a simplistic picture of HPA axis regulation, increased corticosterone should be accompanied by increased ACTH. However, the two hormones need not coincide in time. That basal and stress-induced ACTH were apparently not affected by maternal deprivation at pnd 3 is in contradiction to the results of maternal deprivation on pnd 8 [23]. This might be an age-dependent effect, however, other explanations seem reasonable as well. First, in the pnd 8 study [23], the peak response of ACTH was at 10 minutes and had returned to baseline at 30 minutes. Thus, we might have missed the peak by measuring ACTH at the 30 minutes time point. Secondly, we previously also suggested a decreased expression or processing of the ACTH precursor pro-opiomelanocortin (POMC) [22, 23]. While maternal deprivation did not affect ACTH concentrations whereas corticosterone was increased, an enhancement of adrenal sensitivity appears to be the most likely explanation [13, 17, 23, 25, 26]. The reduced expression of CRH and GR mRNA is either a consequence of high corticosterone exposure [7, 18], but may also be a compensatory response to control and reduce further corticosterone secretion.

Compared to GR mRNA, that during the SHRP was only detectable in the CA1 area of the hippocampus in the mouse, MR mRNA expression was much stronger and clearly expressed in all hippocampal areas and throughout the SHRP [20]. However, neither GR nor MR mRNA expressions in the hippocampus were changed after 24 hours of maternal deprivation, while both were lower in the pnd 8 study [23, 24]. On pnd 3, expression of GRmRNA in the CA1 was 50% lower than on pnd 8 [20] and may be not sensitive to further downregulation. Similarly, MR mRNA expression was not affected in any of the hippocampal subregions, whereas maternal deprivation induced a specific downregulation of MR mRNA expression in the CA2 area at pnd 8 [23, 24]. Interestingly, Vazquez *et al.* [34] reported a similar age-related effect in rats: the downregulation of hippocampal MR mRNA occurred in rats deprived late, but not early during the SHRP.

Some of the direct effects appear to be consistent across species and age. However, from long-term effects of maternal deprivation we know the complex relation to the age of the pups at deprivation [29, 30, ***Chapter 5***]. Here, we showed that also direct effects of maternal deprivation are age-dependent. The state of maturation of the brain and the HPA axis specifically might underlie this differential responsiveness and contribute to the altered phenotype in adulthood.

6.5.2 HPA axis development after reunion

After reunion with their mother the deprived pups no longer responded to a novelty challenge. Maternal behaviour regulates the responsiveness of the pup's HPA axis. This has been demonstrated first in maternally deprived rat pups, where ACTH-induced corticosterone elevations persisted for at least 2 hours following reunion, but had returned to baseline after 6 hours [17, 26]. Already after 24 hours of reunion, basal corticosterone was below those of control animals and the novelty-induced corticosterone secretion was even more suppressed than in control animals (experiment I: pnds 5, 6 and 7), while basal and stress-induced ACTH were reduced at pnd 5 and remained comparable to control mice from pnd 6 onwards. These findings point towards a sustained lower adrenal sensitivity. Since adrenal sensitivity is controlled by a sympathetic influence via the splanchic nerve, alterations in this system could explain the lower adrenal sensitivity to ACTH [3, 4, 11].

The developmental pattern of central components of the HPA axis on the consecutive days following maternal deprivation showed an unexpected variable pattern. CRH mRNA expression in the PVN was decreased immediately after deprivation and remained at less than 50% of the original expression for at least 24 hours following reunion of mother and pups. At pnd 6, 48 hours after reunion of the pups with their mother, CRH mRNA was upregulated to the level of controls. Surprisingly, at pnd 7 CRH mRNA expression superseded control levels and remained higher than controls until pnd 11. Then it caught up with the normal developmental pattern: a gradual reduction of CRH mRNA expression that started in control mice at pnd 8. Because CRH stimulates ACTH release from the pituitary [9, 18, 32], this temporarily increased expression in deprived animals might have enhanced the basal drive of the HPA axis to eventually restore plasma corticosterone concentrations. However, also low circulating corticosterone could be permissive in this respect, reducing negative feedback via the GR in the PVN and hence allowing CRH mRNA expression levels to increase [7, 18].

Maternal deprivation at pnd 3 resulted in a reduced GR mRNA expression in the PVN at pnd 4 that was returned to control values 24 hours following reunion (pnd 5). CRH mRNA expression only recovered after two days, suggesting that alternative or additional mechanisms are of influence, such as indirect regulation via hippocampal MR and GR [7, 10]. Expression of these two receptors was reduced by high corticosterone concentrations in adult rats [2, 9] and following maternal deprivation [23, 24, 28]. GR mRNA expression in CA1 gradually increased with age. We did not find changes directly after maternal deprivation, but the developmental increase of

GR mRNA was arrested on day 5. Although, GR mRNA expression gradually increased again, it remained behind the levels measured in control animals until at least pnd 7. In early adolescence (pnd 28) GR mRNA expression in the hippocampus of deprived mice was still below control animals [***Chapter 5***]. Unfortunately, we were not able to measure GR mRNA for pnds 8 to 13 to determine if GR mRNA in deprived mice would catch up with control mice or had an oscillating pattern over time.

6.5.3 Emerging from the SHRP

Maternal deprivation did not affect the duration of the SHRP that is defined by pituitary and adrenal responsiveness to stress. Both deprived and control animals started to emerge from the SHRP from pnd 9 onwards. We showed for the first time that this is a gradual process and that HPA axis responsiveness is not induced abruptly. Furthermore, CRH mRNA in the PVN was high during the SHRP and about 50% lower thereafter. Here we demonstrate, that the decrease in CRH mRNA expression is also a gradual process in contrast to the impression of our previous study [20]. Each component of the HPA axis follows its own developmental pattern. The question remains, which genetic program(s) and changes in the brain around pnd 9 cause the (gradual) activation of the HPA axis resulting in increased ACTH and corticosterone responses to stress.

6.5.4 Apparent discrepancies

In experiment I, ACTH concentrations immediately after maternal deprivation at pnd 4 were similar to those observed for control animals, which is also seen in rats [31, 34]. Surprisingly, at pnd 5 and 24 hours after reunion with the mother basal ACTH was lower than in control animals. Experiment II revealed that ACTH was elevated directly after deprivation at pnd 4, similar to the results of pnd 8 deprived mice [23]. One reason for these contradictory findings is a possible rebound effect. Schmidt *et al.* [21] demonstrated that during 24 hours of maternal deprivation at pnd 8 ACTH concentrations increased significantly between 8 and 12 hours, but thereafter decreased again to approximately control levels. With 8 hours of maternal deprivation at pnd 3 we observed that ACTH was increased [***Chapter 2***]. Concomitantly, the variation in ACTH levels found at pnd 4 can be explained by measuring at a break-even point towards decreasing levels at pnd 5. Whether the observed lower ACTH concentrations on pnd 5 are caused by depletion of readily available ACTH or due to active maternal behaviour suppressing ACTH release from the pituitary remains to be investigated. These data underline again that maternal deprivation has a strong impact on development with an up to now unknown time frame.

6.5.5 Conclusions

The immediate effects of maternal deprivation on pnd 3 partly resemble those observed for maternal deprivation at pnd 8. Differences are the absence of changes in hippocampal MR and GR mRNA expression. GR mRNA shows a delayed maturation pattern rather than an immediate reduction of expression. Most of the robust effects on central HPA axis parameters gradually re-adjust during the following days, returning to control levels each at its own pace. Since the

immediate effects of maternal deprivation are partially age-dependent, we conclude that the stage of brain development will determine the susceptibility and the regeneration capacity of the various brain systems to environmental disturbances. Whether this is a specific characteristic of mouse genetics and which part of the maternal behaviour contributes to the recovery processes remains to be elucidated.

6.6 Acknowledgements

This study was supported by the Royal Netherlands Academy of Arts and Sciences, NWO–NDRF/STIGON #014-80-005 and NWO #015.01.076.

6.7 References

1. Ader R (1969) Early experiences accelerate maturation of the 24-hour adrenocortical rhythm. Science 163(872): 1225-1226.
2. Bakker JM, van Bel F and Heijnen CJ (2001) Neonatal glucocorticoids and the developing brain: short-term treatment with life-long consequences? Trends Neurosci. 24(11): 649-653.
3. Buijs RM, Wortel J, van Heerikhuize JJ, Feenstra MG, ter Horst GJ, Romijn HJ and Kalsbeek A (1999) Anatomical and functional demonstration of a multisynaptic suprachiasmatic nucleus adrenal (cortex) pathway. Eur. J. Neurosci. 11(5): 1535-1544.
4. Buijs RM, Wortel J, van Heerikhuize JJ and Kalsbeek A (1997) Novel environment induced inhibition of corticosterone secretion: physiological evidence for a suprachiasmatic nucleus mediated neuronal hypothalamo-adrenal cortex pathway. Brain Res. 758(1-2): 229-236.
5. Checkley S (1996) The neuroendocrinology of depression and chronic stress. Br. Med. Bull. 52(3): 597-617.
6. Cirulli F, Santucci D, Laviola G, Alleva E and Levine S (1994) Behavioral and hormonal responses to stress in the newborn mouse: effects of maternal deprivation and chlordiazepoxide. Dev. Psychobiol. 27(5): 301-316.
7. de Kloet ER, Vreugdenhil E, Oitzl MS and Joels M (1998) Brain corticosteroid receptor balance in health and disease. Endocr. Rev. 19(3): 269-301.
8. Dent GW, Smith MA and Levine S (2000) Rapid induction of corticotropin-releasing hormone gene transcription in the paraventricular nucleus of the developing rat. Endocrinology 141(5): 1593-1598.
9. Edwards HE and Burnham WM (2001) The impact of corticosteroids on the developing animal. Pediatr. Res. 50(4): 433-440.
10. Jacobson L, Akana SF, Cascio CS, Shinsako J and Dallman MF (1988) Circadian variations in plasma corticosterone permit normal termination of adrenocorticotropin responses to stress. Endocrinology 122(4): 1343-1348.
11. Jasper MS and Engeland WC (1997) Splanchnicotomy increases adrenal sensitivity to ACTH in nonstressed rats. Am. J. Physiol. 273(2 Pt 1): E363-E368.
12. Levine S (1994) The ontogeny of the hypothalamic-pituitary-adrenal axis. The influence of maternal factors. Ann. N. Y. Acad. Sci. 746: 275-288.
13. Levine S, Huchton DM, Wiener SG and Rosenfeld P (1991) Time course of the effect of maternal deprivation on the hypothalamic-pituitary-adrenal axis in the infant rat. Dev.

Psychobiol. 24(8): 547-558.
14. Makino S, Smith MA and Gold PW (1995) Increased expression of corticotropin-releasing hormone and vasopressin messenger ribonucleic acid (mRNA) in the hypothalamic paraventricular nucleus during repeated stress: association with reduction in glucocorticoid receptor mRNA levels. Endocrinology 136(8): 3299-3309.
15. Matthews SG (2002) Early programming of the hypothalamo-pituitary-adrenal axis. Trends Endocrinol. Metab. 13(9): 373-380.
16. Oitzl MS, Workel JO, Fluttert M, Frosch F and de Kloet ER (2000) Maternal deprivation affects behaviour from youth to senescence: amplification of individual differences in spatial learning and memory in senescent Brown Norway rats. Eur. J. Neurosci. 12(10): 3771-3780.
17. Rosenfeld P, Gutierrez YA, Martin AM, Mallett HA, Alleva E and Levine S (1991) Maternal regulation of the adrenocortical response in preweanling rats. Physiol. Behav. 50(4): 661-671.
18. Sapolsky RM and Meaney MJ (1986) Maturation of the adrenocortical stress response: neuroendocrine control mechanisms and the stress hyporesponsive period. Brain Res. 396(1): 64-76.
19. Schapiro S, Geller E and Eiduson S (1962) Neonatal adrenal cortical response to stress and vasopressin. Proc. Soc. Exp. Biol. Med. 109: 937-941.
20. Schmidt MV, Enthoven L, van der Mark M, Levine S, de Kloet ER and Oitzl MS (2003) The postnatal development of the hypothalamic-pituitary-adrenal axis in the mouse. Int. J. Dev. Neurosci. 21(3): 125-132.
21. Schmidt MV, Enthoven L, van Woezik JH, Levine S, de Kloet ER and Oitzl MS (2004) The dynamics of the hypothalamic-pituitary-adrenal axis during maternal deprivation. J. Neuroendocrinol. 16(1): 52-57.
22. Schmidt MV, Levine S, Oitzl MS, van der Mark M, Mueller MB, Holsboer F and de Kloet ER (2005) Glucocorticoid receptor blockade disinhibits pituitary-adrenal activity during the stress hyporesponsive period of the mouse. Endocrinology 146(3): 1458-1464.
23. Schmidt MV, Oitzl MS, Levine S and de Kloet ER (2002) The HPA system during the postnatal development of CD1 mice and the effects of maternal deprivation. Brain Res. Dev. Brain Res. 139(1): 39-49.
24. Schmidt MV, Oitzl MS, Mueller MB, Ohl F, Wurst W, Holsboer F, Levine S and de Kloet ER (2003) Regulation of the developing hypothalamic-pituitary-adrenal axis in corticotropin releasing hormone receptor 1-deficient mice. Neuroscience 119(2): 589-595.
25. Stanton ME, Gutierrez YR and Levine S (1988) Maternal deprivation potentiates pituitary-adrenal stress responses in infant rats. Behav. Neurosci. 102(5): 692-700.
26. Stanton ME and Levine S (1990) Inhibition of infant glucocorticoid stress response: specific role of maternal cues. Dev. Psychobiol. 23(5): 411-426.
27. Suchecki D, Duarte PB and Tufik S (2000) Pituitary-adrenal axis and behavioural responses of maternally deprived juvenile rats to the open field. Behav. Brain Res. 111(1-2): 99-106.
28. Sutanto W, Rosenfeld P, de Kloet ER and Levine S (1996) Long-term effects of neonatal maternal deprivation and ACTH on hippocampal mineralocorticoid and glucocorticoid receptors. Brain Res. Dev. Brain Res. 92(2): 156-163.
29. van Oers HJ, de Kloet ER and Levine S (1998) Early vs. late maternal deprivation differentially alters the endocrine and hypothalamic responses to stress. Brain Res. Dev. Brain Res. 111(2): 245-252.
30. van Oers HJ, de Kloet ER and Levine S (1999) Persistent effects of maternal deprivation on HPA regulation can be reversed by feeding and stroking, but not by dexamethasone.

J. Neuroendocrinol. 11(8): 581-588.
31. van Oers HJ, de Kloet ER, Whelan T and Levine S (1998) Maternal deprivation effect on the infant's neural stress markers is reversed by tactile stimulation and feeding but not by suppressing corticosterone. J. Neurosci. 18(23): 10171-10179.
32. Vazquez DM (1998) Stress and the developing limbic-hypothalamic-pituitary-adrenal axis. Psychoneuroendocrinology 23(7): 663-700.
33. Vazquez DM and Akil H (1992) Development of pituitary pro-opiomelanocortin gene and peptide expression: characterization and effect of repeated intermittent maternal isolation. Neuroendocrinology 56(3): 320-330.
34. Vazquez DM, van Oers HJ, Levine S and Akil H (1996) Regulation of glucocorticoid and mineralocorticoid receptor mRNAs in the hippocampus of the maternally deprived infant rat. Brain Res. 731(1-2): 79-90.
35. Workel JO, Oitzl MS, Fluttert M, Lesscher H, Karssen AM and de Kloet ER (2001) Differential and age-dependent effects of maternal deprivation on the hypothalamic-pituitary-adrenal axis of brown norway rats from youth to senescence. J. Neuroendocrinol. 13(7): 569-580.
36. Workel JO, Oitzl MS, Ledeboer A and de Kloet ER (1997) The Brown Norway rat displays enhanced stress-induced ACTH reactivity at day 18 after 24-h maternal deprivation at day 3. Brain Res. Dev. Brain Res. 103(2): 199-203.