Early life experience : neuroendocrine adaptations to maternal absence

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Chapter 2

The pituitary-adrenal axis of the CD1 mouse infant desensitises to repeated maternal separations, but remains highly responsive to stress

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2.1 Abstract

Previous studies have shown that a single episode of 8 hours separation from the mother produces in the mouse pup a profound hypothalamic-pituitary-adrenal (HPA) response. In this study we examined in CD1 mice whether repeated daily bouts of 8 hours maternal separation would result in a persistent elevation of corticosterone. For this purpose the effect of repeated 8 hours separations from postnatal days 3 to 5 was measured on basal and stress-induced levels of ACTH and corticosterone as well as on the expression of HPA markers and *c-fos* mRNA in the brain. Circulating levels of glucose and ghrelin were also measured.

The data show that the infant's initial immediate HPA response to 8 hours separation was eliminated when maternal separations are repeated the next two days. Despite the absence of an HPA response to repeated separations, the maternally-deprived mouse continued to respond to novelty exposure. If the repeated maternal separations were combined each time with novelty exposure the response of corticosterone secretion relative to ACTH was enhanced. These effects of separation on the HPA axis were reflected by *c-fos* mRNA expression in the paraventricular nucleus of the hypothalamus (PVN), but not in cortex or thalamus; *c-fos* mRNA in the PVN showed a profound response to a single separation and subsequent desensitisation to repeated separations, but remained responsive to novelty exposure. Basal circulating levels of corticosterone and ACTH were persistently suppressed 16 hours after separation, an effect that also transiently occurred for CRH mRNA in the PVN. Pituitary POMC mRNA and the glucocorticoid receptor (GR) mRNA expression in hippocampus or hypothalamus did not change, but the mineralocorticoid receptor (MR) mRNA expression in the hippocampal dentate gyrus showed a progressive increase with repeated separations. Circulating ghrelin increased and glucose levels decreased after the single as well as the third separation and thus did not reflect the desensitisation of the corticosterone response.

In conclusion, while the infant's initial HPA axis response to repeated maternal absence readily desensitises and a state of hypocorticism is produced, the HPA axis remains highly responsive to mild stressors.

2.2 Introduction

During early postnatal life rats and mice have a stress hypo-responsive period (SHRP, postnatal day (pnd) 4-14 in rats [15, 36, 50] and pnd 1-12 in mice [38]). This period is characterised by stable and low circulating basal levels of corticosterone and most (mild) stressors that trigger a profound ACTH and corticosterone response at adulthood do so only weakly during the SHRP. The hypo-responsiveness of the HPA axis is due to the presence of the mother [3, 16], which can be demonstrated by separation of pups from maternal care for a prolonged period of time. After, for example, a single 24 hours maternal deprivation, basal levels of ACTH and corticosterone are increased. Moreover, the secretion of pituitary-adrenal hormones has now become responsive to

mild stressors, resulting in a further rise in plasma ACTH and corticosterone [31, 42, 44]. These neuroendocrine effects in response to maternal separation are already detectable as early as after 4 hours of maternal separation, but significantly greater at 8 and 24 hours [17, 31, 39].

Ever since adverse early life events have been identified as a major risk factor for the development of depression and anxiety disorders in humans [2, 11, 12], rodents separated as pups from maternal care have been widely used as laboratory model to study the underlying mechanism [14, 32]. In particular, the brief daily separation of mother and pups, a procedure called handling, was shown to result in a persistent attenuation of HPA responsiveness in later life, presumably through a mechanism involving maternal care effects on methylation of the glucocorticoid receptor (GR) in the developing brain [34, 49]. In contrast, repeated separations for approximately 3 hours and longer usually were shown to produce increased HPA axis responsiveness and behavioural fearfulness in adult rats [7, 18, 20, 23, 27] and mice [19, 28, 29, 47].

The mechanism underlying these lasting effects of prolonged daily separations is still poorly understood. Maternal care is important, but cannot be the only factor [20, 21]. Daily activation of the HPA axis, as induced by repeated and prolonged separation of mother and pups, has indeed been considered an additional factor, because it will result in exposure to increased levels of corticosterone, particularly after experience of a stressor [22]. In support of a role for corticosterone, this hormone appeared crucial for maturation of neuronal circuitry involved in processing odor fear conditioning at a time during the SHRP that hormone concentrations normally are stable and low. Infants readily learn maternal odor to support attachment behaviour, but if exposed to exogenous corticosterone in the amygdala the infant can switch towards avoidance behaviour [25, 26]. It is therefore reasonable to assume that in the repeated maternal separation paradigm the HPA axis is activated each time pups are deprived of maternal care with each successive separation period. However, to our knowledge there are no data to support this line of reasoning.

In the current study we tested the hypothesis that daily repeated maternal separations from the mother would sensitise the pup's HPA axis for enhanced secretion of corticosterone. Crucial for testing this hypothesis is an HPA axis activation each time the pups are separated from their mother. Previously we observed in the mouse a profound activation of ACTH and corticosterone after 8 hours of maternal separation [39]. Therefore, we used 8 hours of maternal absence to determine the immediate effects of up to three consecutive daily maternal separations on basal and novelty-induced pituitary-adrenal activity. In addition, we measured with *in situ* hybridisation the expression of POMC mRNA in the anterior pituitary, CRH mRNA, GR mRNA and and *c-fos* mRNA in the PVN, and MR and GR mRNA in the hippocampus. In view of the 8 hours of food deprivation representative metabolic signals, *e.g.* ghrelin and glucose, were also measured. We found that the infant's initial HPA axis response readily desensitised to maternal absence producing lower corticosterone levels than in the non-separated pups, but that its HPA response to novelty persisted.

2.3 Materials and Methods

2.3.1 Animals

Offspring of CD1 mice (obtained from Charles River, The Netherlands) was used. 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; 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 containing sawdust bedding and tissue to provide nest-building material during the last week of gestation. Females were checked for litters daily between 9:00 and 9:30 hours. If litters were present, the day of birth for that litter was then defined as postnatal day 0 (= pnd 0). On the day after parturition, pnd 1, litters were culled to eight healthy pups (four males and four 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. The protocols were approved by the Animal Care Committee of the Faculty of Medicine, Leiden University (Leiden, The Netherlands).

2.3.2 Separation procedures

Mothers nursing litters selected for maternal separation were removed from their cage and placed in clean type 3 polycarbonate cages at 9:00 hours. The home cage containing the pups remained in the adjacent room on a heating pad (30 - 33°C) to control for pup body temperature. After 8 hours (at 17:00 hours), the mothers were reunited with their pups and left undisturbed until the next separation period or until testing. Control litters were left undisturbed.

2.3.3 Novelty exposure

For novelty exposure first the mother was removed from the home cage. Then, in an adjacent room, the pups were individually placed in clean novel cages to induce novelty stress. These novel cages were placed on heating pads (30 - 30°C) to control for pup body temperature. After 30 minutes, the pups were either placed back with their mothers in the home cage or sacrificed.

If novelty exposure took place on a testing day, four pups (two males and two females) were sacrificed immediately after removal of the dam from the home cage. The remaining pups were then transferred to novel cages and sacrificed 30 minutes later for testing. All other procedures remained constant.

2.3.4 Experimental designs

Experiment I: To investigate the immediate, cumulative effects of repeated separations mice were separated once (pnd 3), twice (pnds 3 and 4) or three times (pnds 3, 4 and 5) from maternal care for a period of 8 hours. Before (at 9:00 hours), as well as at the end of each separation session (at 17:00 hours), mice were sacrificed for determination of peripheral and central markers of

the HPA axis. At pnd 3 one group of animals served as basal measurement for both the non-separated and first time separated animals. Non-separated animals were only measured at 9:00 hours, since no circadian effects were detected for corticosterone in "Experiment I". The fixed factors were TREATMENT (non-separated; separated (basal); separated (+8 hours)) and AGE (pnds 3, 4 and 5).

Experiment II: To test whether pups at pnd 5 exhibited an adult-like response to the third separation session, at 9:00 hours mothers were removed from their nests to initiate maternal separation and the home cages containing the litters were placed on a heating pad. Thereafter, every hour blood samples were taken up to 8 hours of maternal absence. For this purpose, to minimise nest effects, from each litter four pups (two males and two females) were sacrificed at two different time points. At 9:00 hours (basal time point) one group of animals served as a basal measurement for both non-separated and first time separated animals. Fixed factors were TREATMENT (non-separated at pnd 5; single separation at pnd 5; triple separation at pnds 3, 4 and 5, tested at pnd 5) and TIME (basal (=0 hours) and 1, 2, 3, 4, 5, 6, 7 and 8 hours of maternal separation).

Experiment III: To determine whether the desensitisation to maternal separation observed in "Experiment I and II" was an age-specific effect we repeated this experiment, but this time included an 8 hours maternal separation applied to separate groups of naïve animals at pnds 4 and 5. On test days, four pups from each litter (two males and two females) were sacrificed immediately providing a basal sample (at 9:00 hours). The other four pups were sacrificed after 8 hours of maternal separation (at 17:00). The fixed factors were TREATMENT (single separation at pnds 3, 4 or 5; double separation at pnds 3 and 4, tested at pnd 4; triple separations at pnd 3, 4 and 5, tested at pnd 5) and TIME (basal and 8 hours maternal separation).

Experiment IV was designed to test whether pups were able to respond to novelty stress with increased corticosterone and ACTH directly after a first, second or third period of separation. On test days four pups from each nest (two males and two females) were sacrificed 8 hours after maternal separation, providing a "separated" sample (at 17:00 hours). The remaining pups were then exposed to novelty and sacrificed 30 minutes later. Fixed factors were TREATMENT (non-separated at pnd 5; single separation at pnd 5; triple separation at pnds 3, 4 and 5, tested at pnd 5) and TIME (basal, either at 9:00 hours or after 8 hours of maternal separation at 17:00 hours; 30 minutes individual novelty exposure).

Experiment V: The objectives were: (1) to determine the response of corticosterone, ghrelin and glucose under the conditions of desensitisation to repeated maternal separations and (2) to determine activation of central hypothalamic brain areas in response to maternal separation and novelty by measuring *c-fos* mRNA expression. Pups were sacrificed after the first or the third period of 8 hours of separation from the dam in the absence or presence of additional novelty exposure. Non-separated animals sacrificed at these time points served as basal controls. Plasma corticosterone levels were determined in all these groups. Blood glucose and ghrelin levels were measured only under basal conditions and after 8 hours of maternal absence. Fixed factors were

then TREATMENT (single separation at pnd 5 and triple separation at pnds 3, 4 and 5, tested at pnd 5) and TIME (basal and separated). Expression levels of *c-fos* and CRH mRNA were measured in the PVN and of NPY mRNA in the arcuate nucleus in all treatment groups. Fixed factors, similar as for corticosterone, were then TREATMENT (1st SEP and 3rd SEP) and TIME (basal, separated, novelty).

2.3.5 Collection of blood plasma and brains

At specified time points described in Experiments I to IV (see "Experimental designs"), animals were sacrificed by decapitation and trunk blood was collected individually in 1.5 ml EDTA-coated microcentrifuge tubes. Blood samples were kept on ice and centrifuged for 15 minutes at 13000 rpm at 4°C. Plasma was then transferred to 1.5 ml Eppendorf tubes and stored frozen at -20°C until determination of corticosterone and ACTH concentrations.

Blood glucose levels were measured (Accu-Check Compact, Roche, Germany) using a droplet of trunk blood left on the head or body. After decapitation whole heads, of which skin and jaws were removed, were snap frozen in isopentane on dry ice and stored at -80°C for *in situ* hybridisation.

2.3.6 Hormone analysis

Per experiment plasma corticosterone and ACTH concentrations were measured separately using commercially available radio immunoassay (RIA) kits containing ^{125}Iodine labelled corticosterone or ACTH, respectively (MP Biomedicals Inc., USA). Corticosterone concentrations were determined in duplicate from an extended standard curve (0, 6.25, 12.5, 25, 50, 100, 250, 500 and 1000 ng corticosterone/ml), since we noted that the lower boundary provided by the kit was not sensitive enough to measure basal plasma concentrations. ACTH samples were determined in a 50% dilution, starting with 25 µl blood plasma.

Plasma levels of ghrelin were measured using a commercially available RIA kit containing ^{125}Iodine labelled ghrelin (Linco Research, USA). Ghrelin concentrations were determined in a 1:4 dilution, starting with 25 µl blood plasma. Vials for each RIA were counted for 2 minutes in a gamma-scintillation counter (Packard Minaxi Gamma counter, Series 5000).

2.3.7 In situ *hybridisation*

Brains were sectioned at -20°C in a cryostat microtome at 16 µm in the coronal plane at the level of the PVN, pituitary and dorsal hippocampus. Sections were thaw-mounted on poly-L-lysine (0.01%) coated slides, air-dried and kept at -80°C.

In situ hybridisations were performed using ^{35}Sulphur labelled ribonucleotide probes for corticotrophin releasing hormone (CRH; rat full length coding region; measured in PVN), glucocorticoid receptor (GR; mouse exon 2 fragment; measured in PVN, hippocampus and pituitary), mineralocorticoid receptor (MR; mouse exon 2 region; measured in hippocampus) and pro-opiomelanocortin (POMC; mouse 0.9 kb fragment; measured in pituitary). Sections were fixed in 4% paraformaldehyde/0.5% glutaraldehyde and thereafter 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 brain sections per slide) were saturated with 100 µl of 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}Sulphur labelled ribonucleotide probe. Brain sections were coverslipped and incubated overnight at 55°C. The following day the sections were rinsed in 2 × SSC, treated with RNAse A (20 mg/ml) 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.
For determining *c-fos* mRNA expression (PVN) *in situ* hybridisations using ^{33}Phosphor labelled oligonucleotide probes were performed as described previously [24]. 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.

2.3.8 Statistical analysis

In each experiment every treatment consisted of three litters per age group (n=12). Data were analysed by analysis of variance (ANOVA). The level of significance was set at P<0.05. When appropriate this was followed by Tukey's *post hoc* comparisons. The initial analyses in each experiment included sex as a factor, but because it was not a significant factor in any experiment, data were collapsed across this variable. Data are presented as mean ± S.E.M.

2.4 Results

2.4.1 In ***Experiment I*** the immediate (cumulative) effects were investigated of one (pnd 3), two (pnds 3 and 4) or three (pnds 3, 4 and 5) times 8 hours daily maternal separation(s) on the basal development of both central and peripheral HPA axis markers.

2.4.1.1 Corticosterone (***Figure 2.1.A***)

We observed a main affect of treatment (F(2,48)9.58; P<0.001) and an interaction between age and treatment (F(3,48)6.18; P<0.001), indicating that the effect of additional separations (treatment) depended on the previous daily experiences of the animals. The first period of separation at pnd 3 resulted in a robust increase in corticosterone (P<0.01). After the second (pnd 4) and third (pnd 5) period of separation this corticosterone increase was completely abolished. Basal corticosterone the next morning 16 hours after each consecutive separation (at pnds 4 and 5) was significantly lower compared to non-separated animals (P<0.01).

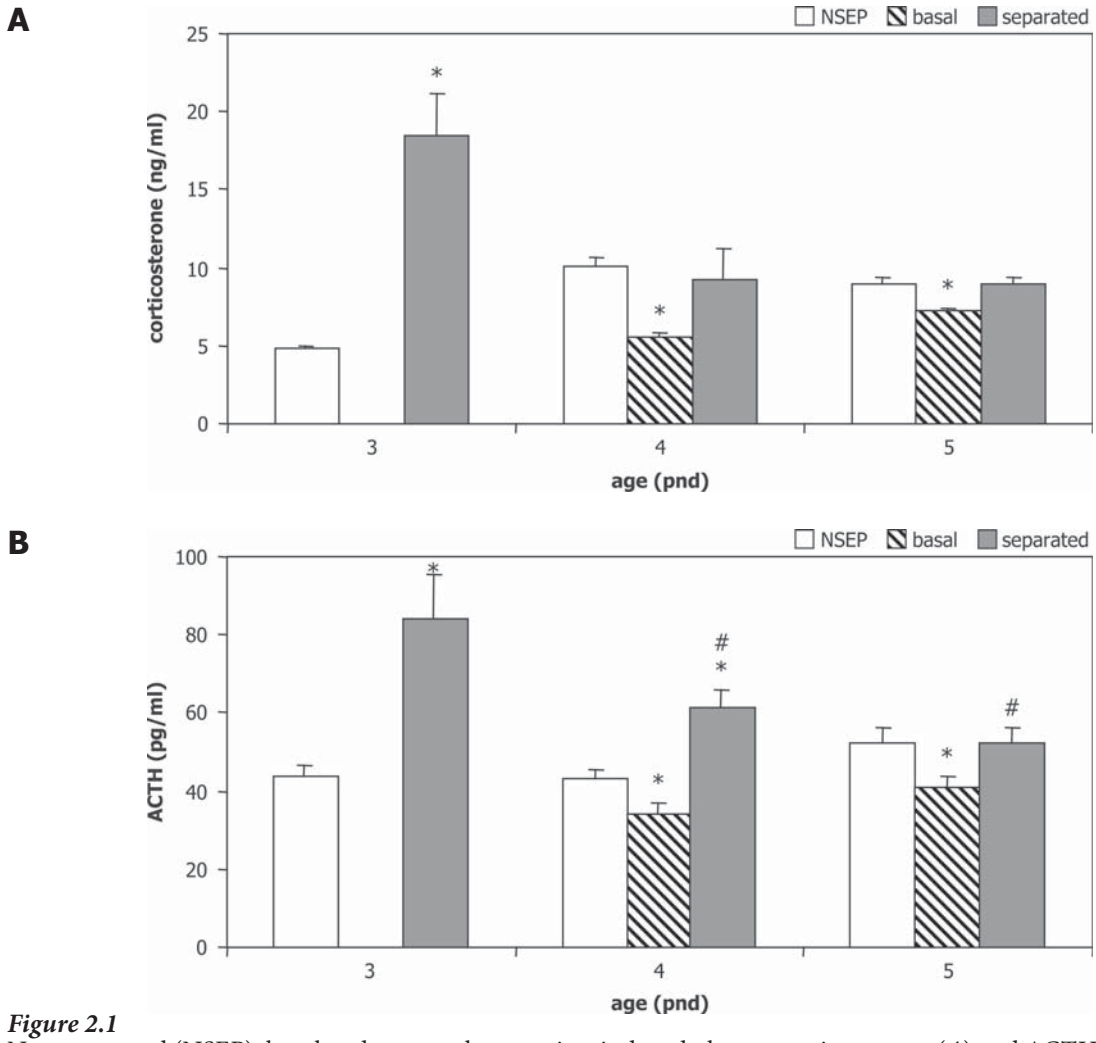

Figure 2.1
Non-separated (NSEP), basal and maternal separation-induced plasma corticosterone (***A***) and ACTH (***B***) levels in mouse pups tested at different ages (non-separated at 9:00 hours; basal: basal levels at 9:00 hours of pups maternally separated for 8 hours on the preceding day(s); separated: separation induced levels at 17:00 hours of animals separated for 8 hours). Maternal separation took place at pnds 3, 4 and 5 resulting in mice that were separated for the first time at pnd 3, for a second time at pnd 4, and for a third time at pnd 5. The control group at pnd 3 also serves as a 'basal' group for maternal separation at this age. Data represent mean ± S.E.M., * $P<0.05$ (significant from "non-separated" at the same day), # $P<0.05$ (significant from "basal").

2.4.1.2 ACTH (***Figure 2.1.B***)

We observed a main effect of treatment ($F(2.48)16.99$; $P<0.001$). The first period of separation at pnd 3 resulted in a robust increase in ACTH ($P<0.001$). After the second period of separation at pnd 4 ACTH was increased compared to both basal levels ($P<0.01$) and non-separated animals ($P<0.01$), although the response was less than at pnd 3. The third period of separation (pnd 5) did not differ from non-separated animals. The magnitude of the response was smaller than after a first (pnd 3) or second (pnd 4) period of separation compared to basal ACTH. Basal ACTH was significantly lower after each consecutive period of separation compared to non-separated animals (at pnds 4 and 5; $P<0.05$).

2.4.1.3 CRH mRNA in PVN (***Table 2.1***)

CRH mRNA expression levels increased with age ($F(2,48)6.42$; $P<0.01$). After the first separation

period at pnd 4 basal levels of CRH mRNA expression were decreased compared to non-separated animals ($P<0.05$), but after a second period of deprivation (pnd 4, basal pnd 5), this difference was abolished.

2.4.1.4 POMC mRNA in pituitary (*Table 2.1*)

Neither basal, nor separation-induced levels of POMC mRNA expression in the pituitary were affected by maternal separation, irrespective of the number of repetitive separations.

2.4.1.5 MR mRNA in hippocampus (*Table 2.1*)

MR mRNA expression was measured in the CA1, CA2, CA3-4 and dentate gyrus (DG) hippocampal subfields. We detected subfield specific effects: in the CA3-4 area maternal separation did not affect expression, neither after 8 hours nor at basal levels. We observed a main effect of age in the CA2 ($F(2,48)5.10$; $P<0.05$) and DG ($F(2,48)5.12$; $P<0.05$) and an interaction between

Table 2.1: Expression of mRNA for central HPA axis markers in selected brain regions

			pnd 3	pnd 4	pnd 5
CRH	PVN	NSEP	35.34 ±2.72	40.57 ±2.59	43.88 ±2.19
		basal		**31.92** ±1.28*	43.46 ±1.65
		separated	34.00 ±3.29	35.32 ±1.55	41.43 ±3.07
POMC	pituitary	NSEP	46.16 ±1.75	47.53 ±3.24	46.08 ±1.64
		basal		49.14 ±2.36	47.96 ±2.82
		separated	44.81 ±1.51	43.17 ±2.12	47.18 ±2.20
MR	hippocampus CA1	NSEP	39.99 ±2.09	47.81 ±4.18	42.39 ±2.66
		basal		44.29 ±2.09	**50.62** ±3.63*
		separated	41.46 ±3.49	41.52 ±2.46	45.10 ±2.37
	hippocampus CA2	NSEP	95.01 ±4.10	107.56 ±3.80	98.64 ±5.54
		basal		103.96 ±2.88	111.26 ±3.15
		separated	103.02 ±2.15	111.49 ±4.95	112.44 ±5.72
	hippocampus CA3-4	NSEP	58.21 ±3.24	62.74 ±2.71	58.07 ±3.71
		basal		59.57 ±1.70	63.36 ±3.23
		separated	58.59 ±1.43	64.06 ±4.52	63.24 ±3.84
	hippocampus DG	NSEP	57.35 ±2.95	65.53 ±3.92	68.85 ±3.19
		basal		64.93 ±1.91	69.82 ±3.37
		separated	65.42 ±2.00	70.28 ±4.93	**80.88** ±3.39*#
GR	PVN	NSEP	37.73 ±2.13	39.68 ±2.17	39.11 ±1.97
		basal		44.77 ±2.39	45.14 ±2.25
		separated	37.10 ±2.13	44.30 ±1.87	39.98 ±1.97
	pituitary	NSEP	59.65 ±1.83	56.67 ±1.08	56.53 ±1.63
		basal		57.18 ±0.83	56.26 ±1.60
		separated	57.83 ±1.06	60.12 ±2.05	58.63 ±1.23
	hippocampus CA1	NSEP	38.42 ±2.49	36.46 ±2.47	38.76 ±2.54
		basal		37.58 ±2.64	40.77 ±2.23
		separated	40.05 ±1.85	40.44 ±5.11	38.70 ±1.82

Non-separated (NSEP), basal and maternal separation induced mRNA expression levels of central HPA axis markers in mouse pups tested at different ages. (See ***Figure 2.1*** for an explanation of the different treatment groups.) Relative optical density levels of mRNA expression are expressed in arbitrary units. All data presented as mean ± S.E.M., * $P<0.05$ (significant from "NSEP" at the same day), # $P=0.06$ (versus "basal" at the same day).

age and treatment in the CA1 area (F(2,48)2.80; $P<0.05$). However, only in the DG after the third period of separation (at pnd 5) MR mRNA expression significantly increased compared to non-separated animals ($P<0.05$). There was also a trend towards a significant increase compared to basal levels ($P=0.06$). Basal MR mRNA expression after each successive period of separation was not affected.

2.4.1.6 GR mRNA in PVN, pituitary and hippocampus (***Table 2.1***)
In the PVN and the pituitary, consecutive 8 hours maternal separations at pnds 3, 4 and 5 did not lead to an altered GR mRNA expression, neither did it affect basal expression.

In the hippocampus we were only able to measure GR mRNA expression in the CA1 subfield at these ages (pnds 3–5, see also: [38]). In the CA3 and DG subfields of the hippocampus GR mRNA expression was very low and remained below the detection limit. GR mRNA expression was not altered by consecutive periods of maternal separation or age, nor did separation affect basal expression levels.

2.4.2 Experiment II was designed to compare the time course of the effect of a single 8 hours maternal separation at pnd 5 with the outcome of three consecutive daily 8 hours maternal separations at pnds 3, 4 and 5, measured at pnd 5.

2.4.2.1 Corticosterone (***Figure 2.2.A***)
We observed a main effect of treatment (F(2,233)49.18; $P<0.001$), time (F(8,233)16.87; $P<0.001$) and an interaction between treatment and time (F(9,233)13.84; $P<0.001$), indicating that as a consequence of previous experiences of the pups, the time course of corticosterone secretion over 8 hours of maternal absence was changed. The three times separated group no longer responded to separation with an increase in corticosterone and had lower plasma concentrations at 9:00 and 13:00 hours compared to non-separated animals ($P<0.05$) and at all time points compared to first time separated animals ($P<0.05$). On the other hand, animals separated for the first time showed an increase in corticosterone; this increase only started after 5 hours of maternal absence (at 14:00 hours).

Post hoc analyses also showed that corticosterone measured at 17:00 hours in animals separated for the first or the third time were significantly higher that those measured at 9:00 hours ($P<0.01$ within each treatment group).

2.4.2.2 ACTH (***Figure 2.2.B***)
We observed a main effect of treatment (F(2,233)36.63; $P<0.001$), time (F(8,233)15.53; $P<0.001$) and an interaction between treatment and time (F(9,233)6.90; $P<0.001$), indicating that the response to 8 hours maternal separation at pnd 5 depended on the previous experience of the pups. Pups separated for the third time did not differ from non-separated animals at any time point. Animals separated for the first time, however, did show an increase in plasma ACTH

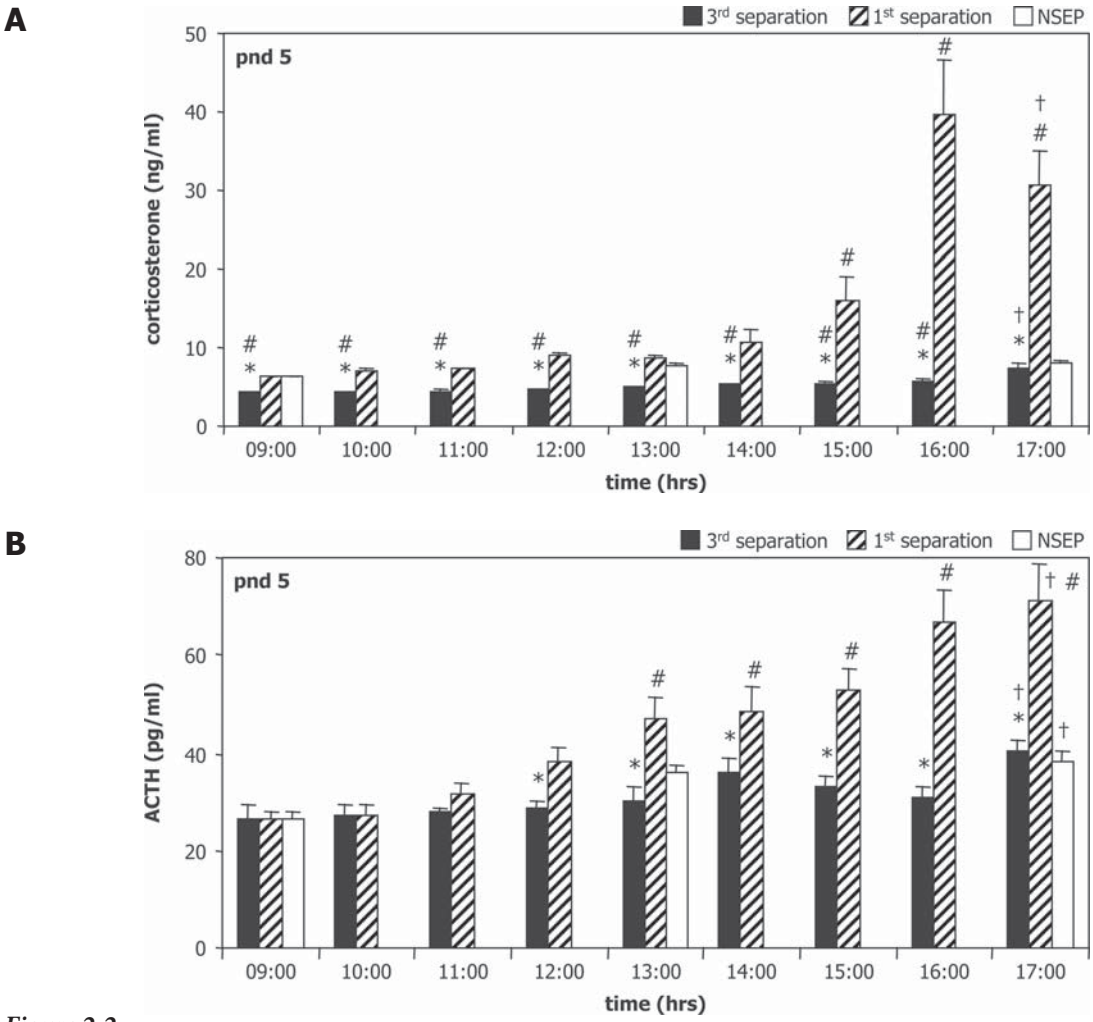

Figure 2.2
Corticosterone (***A***) and ACTH (***B***) response to 8 hours maternal separation at pnd 5 in animals with no previous history of separation (1st separation) and in animals previously exposed to 8 hours separation at pnds 3 and 4 (3rd separation). Non-separated animals (NSEP) were left undisturbed until the moment of testing. At 9:00 hours (start of separation period) one group of animals served as a basal measurement for both the "non-separated" and "1st separation" group. Data represent mean ± S.E.M. * $P<0.05$ (significant from "1st separation"), # $P<0.05$ (significant from (both) closest "non-separated" group(s)), † $P<0.01$ (significant from 9:00 hours, within treatment group).

levels compared to non-separated animals. ACTH started to rise 2 to 3 hours after the onset of separation (at 11:00 – 12:00 hours)

Post hoc analyses also showed that ACTH values measured at 17:00 hours in either group were significantly higher than at 9:00 hours ($P<0.01$ within each treatment group).

2.4.3 Experiment III was designed to determine whether the absence of the endocrine response to a third period of maternal separation as observed in Experiment I was age-specific, by repeating this experiment now including a single maternal separation at pnds 4 and 5.

2.4.3.1 Corticosterone (***Figure 2.3.A***)

We observed a main effect of treatment ($F(4,103)13.16$; $P<0.001$), time ($F(1,103)92.42$; $P<0.001$) and an interaction between treatment and time ($F(4,103)14.74$; $P<0.001$), indicating that the response to maternal separation (time) changed, depending on the amount of consecutive separation periods

(treatment). Irrespective of the age of the animals, pups reacted with an increase in corticosterone to the first period of maternal separation (all ages $P<0.001$). Furthermore, in response to each successive period of separation the magnitude of corticosterone secretion decreased (second separation at pnd 4, $P<0.05$), until it disappeared (third separation at pnd 5).

2.4.3.2 ACTH (***Figure 2.3.B***)

We observed a main effect of treatment ($F(4,103)6.77$; $P<0.001$), time ($F(1,103)60.29$; $P<0.001$) and an interaction between treatment and time ($F(4,103)3.16$; $P<0.05$), indicating that depending on the amount of consecutive separation periods the ACTH response to 8 hours maternal absence changed. To the first time maternal separation we detected an increase in ACTH, irrespective of age (all ages $P<0.001$). Furthermore, to the second period of separation the magnitude of ACTH response decreased (pnd 4, $P<0.001$), whereas a third exposure gave an even smaller, though still significant response (pnd 5, $P<0.05$).

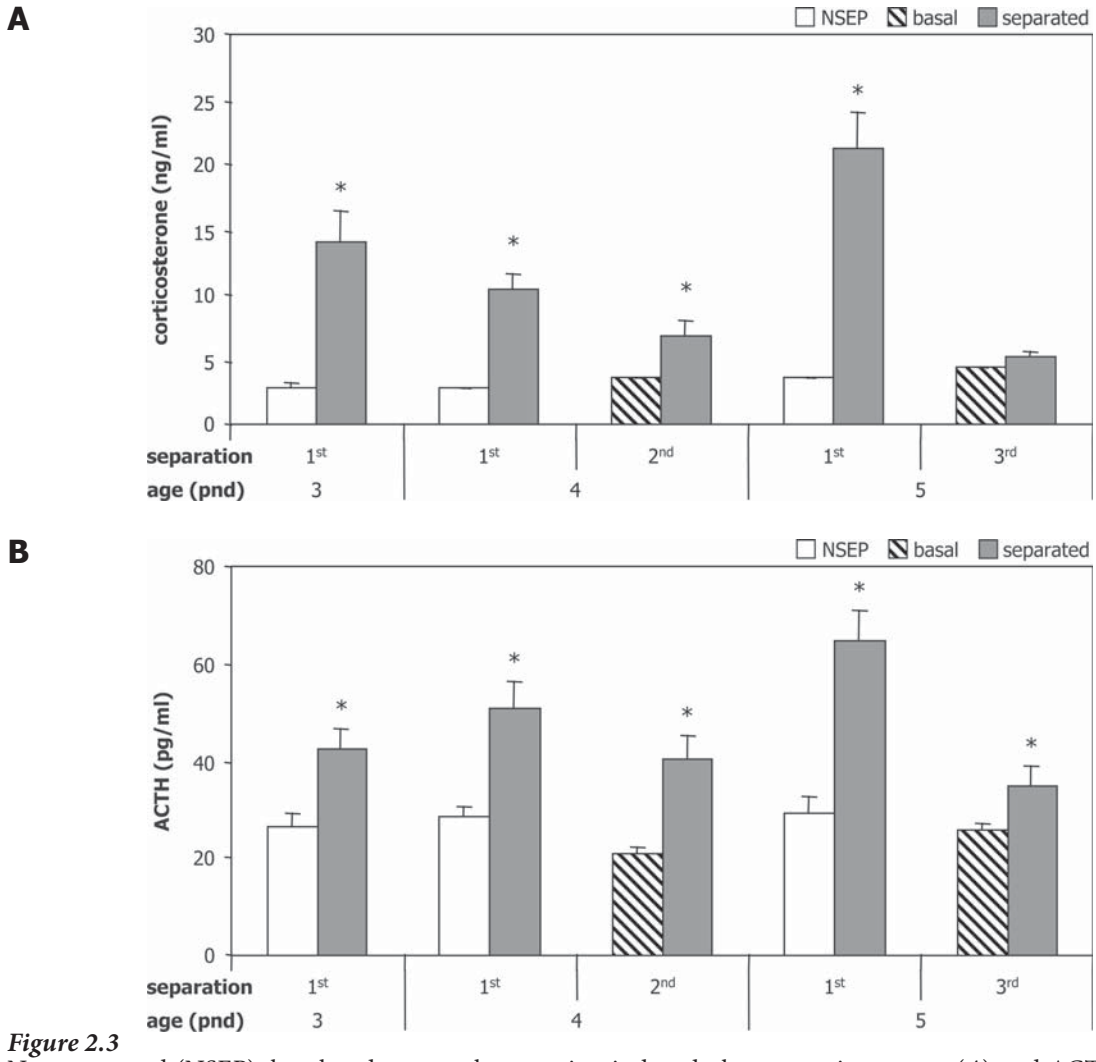

Figure 2.3
Non-separated (NSEP), basal and maternal separation-induced plasma corticosterone (***A***) and ACTH (***B***) levels in mouse pups tested at different ages ("1st": first 8 hours of maternal separation at pnds 3, 4 or 5; "2nd": separated at pnds 3 and 4; "3rd": separated at pnds 3, 4 and 5; see also ***Figure 2.1*** for a detailed explanation on the treatment groups). Data represent mean ± S.E.M., * $P<0.05$, (significant from "NSEP" or "basal" within the same treatment group at the same day).

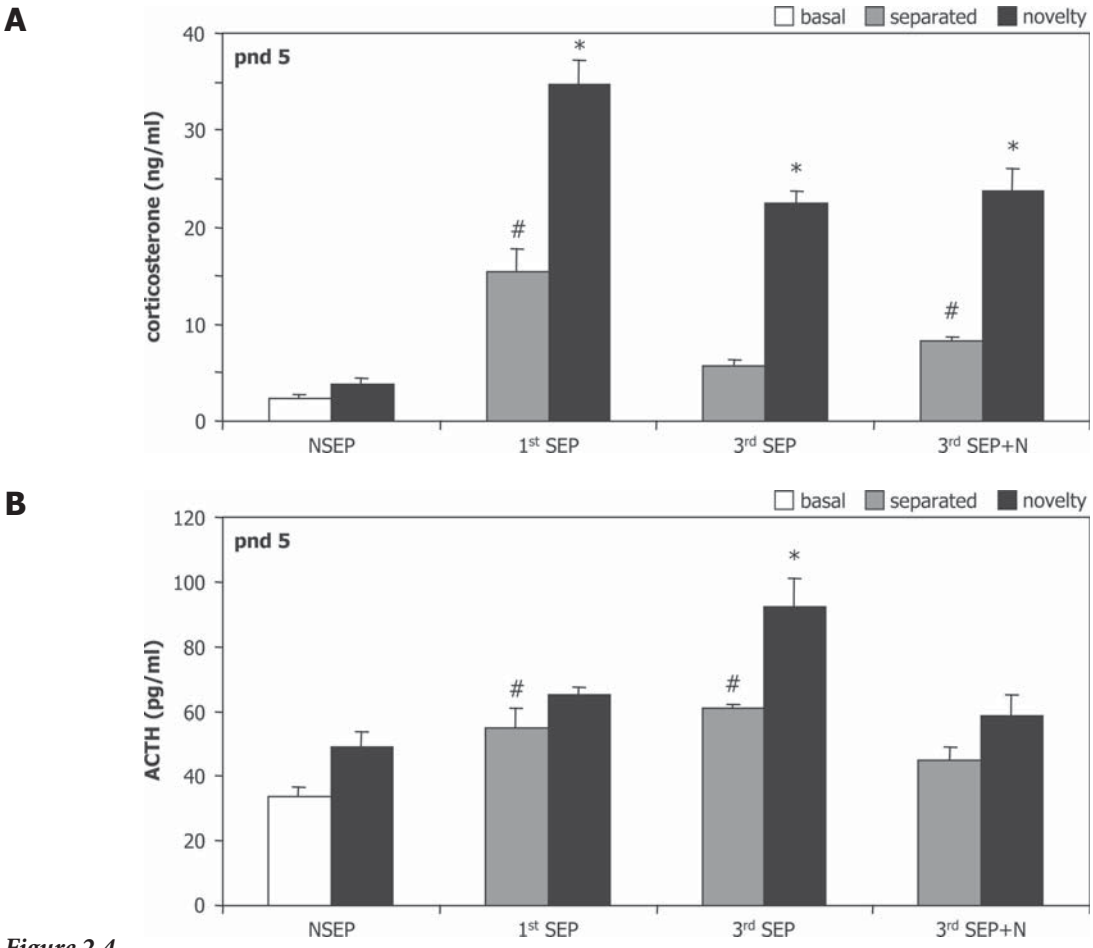

Figure 2.4
Basal, separation and novelty (30 minutes isolated exposure to a new environment) induced plasma corticosterone (***A***) and ACTH (***B***) levels at pnd 5. NSEP and 1st SEP had no previous history of treatments. 3rd SEP animals were exposed to 8 hours separation at pnds 3 and 4. 3rd SEP+N animals were exposed to 8 hours maternal separation directly followed by 30 minutes novelty at pnds 3 and 4. "Separation" levels of 1st SEP, 3rd SEP and 3rd SEP+N were measured at the end of 8 hours maternal separation. Data represent mean ± S.E.M. * $P<0.001$ (significant from basal or separated), # $P<0.05$ (significant from NSEP).

2.4.4 Experiment IV was designed to determine whether corticosterone and ACTH still responded to exposure of an additional novelty stressor directly after maternal separation.

2.4.4.1 Corticosterone (*Figure 2.4.A*)

We observed an effect of treatment ($F(3,77)40.66$; $P<0.001$), time ($F(1,77)69.38$; $P<0.001$) and an interaction between treatment and time ($F(3,77)8.55$; $P<0.001$). Depending on the treatment the pups received, their response to novelty changed. As expected, non-separated animals were not able to respond to novelty with a rise in corticosterone, whereas pups separated from their mother for the first time were able to respond to novelty even though corticosterone levels were already increased due to the preceding separation.

Though pups that were separated for three times had corticosterone levels similar to those observed for untreated animals, they were still capable to respond to novelty. Also pups that were repeatedly separated in combination with novelty exposure after each separation period (3rd SEP+N) still responded to novelty. Their corticosterone levels after the third separation were

significantly higher than those of non-separated animals (NSEP), but not compared to those of the repeated separation group without novelty exposure (3rd SEP).

2.4.4.2 ACTH (***Figure 2.4.B***)

We observed a main effect of treatment ($F(3,77)7.05$; $P<0.001$) and time ($F(1,77)9.11$; $P<0.01$). Although ACTH levels of pups separated for the first time were already higher than those of non-separated pups, animals in both groups were unable to respond to novelty with an increase in ACTH.

Animals separated for three times (3rd SEP) had higher ACTH levels compared to non-separated animals and responded to novelty with a further increase in ACTH. In pups receiving maternal separation in combination with novelty each time (3rd SEP+N) showed, after the third separation period, ACTH levels similar to those of non-separated animals and the exposure to a novelty stressor did not evoke a response.

2.4.5 Experiment V: The objective was to determine circulating ghrelin and glucose concentrations in response to repeated maternal separations. Corticosterone and mRNA expression of *c-fos* mRNA were also measured under basal conditions and in response to maternal separations with and without novelty exposure.

2.4.5.1 Corticosterone (***Figure 2.5***)

A main effect was observed of treatment ($F(1,71)24.92$; $P<0.001$), time ($F(2,71)130.97$; $P<0.001$) and an interaction between these factors ($F(2,71)12.26$; $P<0.001$). In response to a first separation period corticosterone increased ($P<0.001$) and these levels increased even further when pups were subjected to additional novelty stress ($P<0.001$). In response to a third period of separation, however, corticosterone values did not alter, though exposure to a novel environment was able to activate the HPA axis ($P<0.001$). Basal corticosterone at the start of either the first or third separation period did not differ.

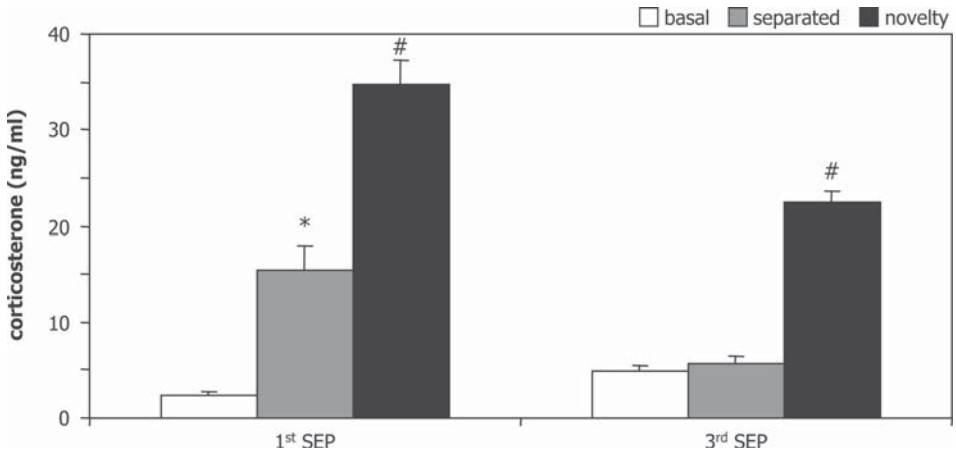

Figure 2.5
Corticosterone in animals at pnd 5 with no previous history of separation (1st SEP) and in animals previously exposed to 8 hours separations at pnds 3 and 4 (3rd SEP). Corticosterone response was measured after 8 hours of maternal separation and after 8 hours of maternal separation with an additional 30 minutes of novelty stress. Data represent mean ± S.E.M. * $P<0.05$ (significant from basal), # $P<0.05$ (significant from separated).

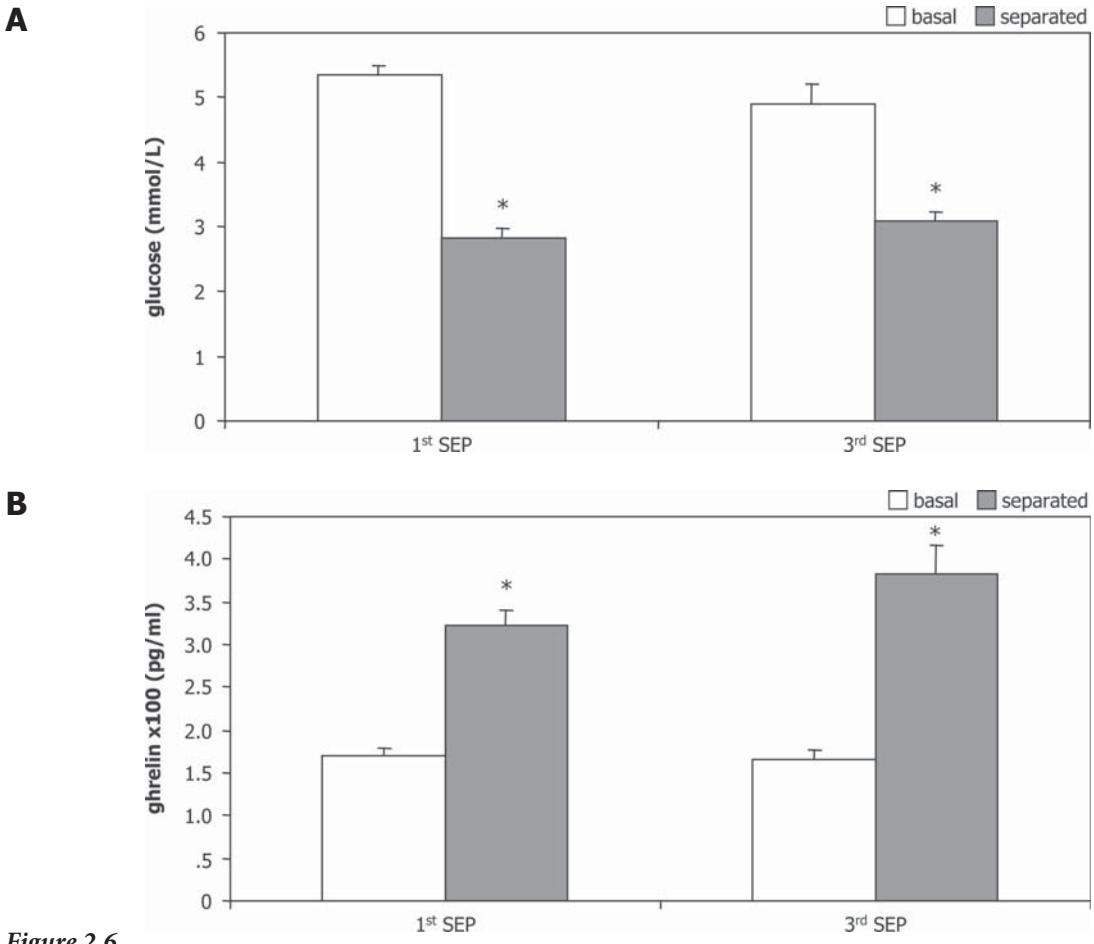

Figure 2.6
Glucose (***A***) and ghrelin (***B***) response to maternal separation at pnd 5 in animals with no previous history of separations (1st SEP) and in animals previously exposed to 8 hours separation at pnds 3 and 4 (3rd SEP). Data represent mean ± S.E.M. * $P<0.05$ (significant from basal).

2.4.5.2 Glucose (*Figure 2.6.A*)

A main effect of time was determined ($F(1,47)115.79$; $P<0.001$), but not of treatment. In response to a first maternal separation period blood glucose levels decreased to almost half their original levels ($P<0.01$). After repeated separations this same response was still present ($P<0.01$). Basal glucose values were similar between the animals separated for the first or third time.

Table 2.2: mRNA expression of *c-fos* in selected brain areas

		basal	separated	novelty
Cortex	1st SEP	14.63 ±2.09	13.92 ±2.22	15.10 ±3.28
	3rd SEP	12.34 ±2.56	11.70 ±2.15	12.00 ±0.92
pPVTh	1st SEP	40.76 ±2.56	39.35 ±4.31	40.08 ±3.61
	3rd SEP	39.76 ±4.50	31.85 ±2.55	32.39 ±1.7

To determine central activation of neurons in response to maternal separation and additional novelty stress *c-fos* mRNA expression was determined in the PVN (***Figure 2.7***), cortex and pPVTh (paraventricular thalamic nucleus). Data are expressed as optical density measured in arbitrary units and presented as mean ± S.E.M.

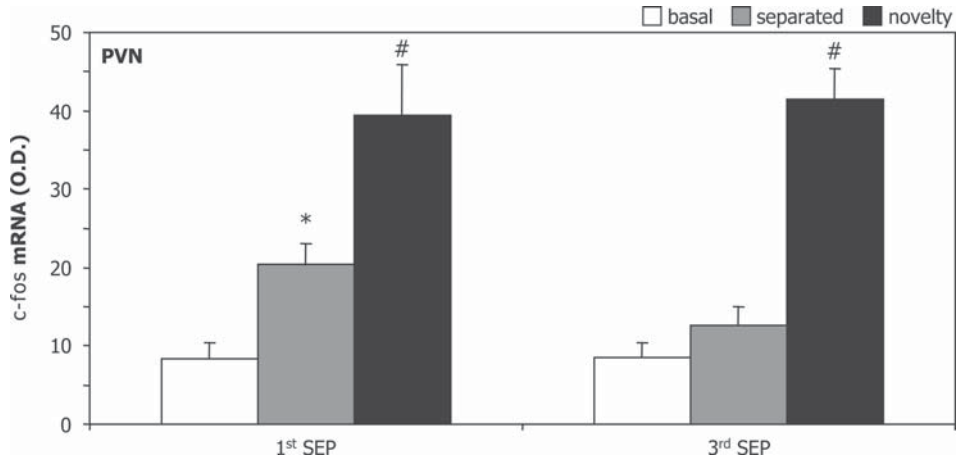

Figure 2.7
C-fos mRNA expression measured in the PVN at pnd 5 in animals with no previous history of separation (1st SEP) and in animals previously exposed to 8 hours separations at pnds 3 and 4 (3rd SEP). Expression was measured after 8 hours of maternal separation and after 8 hours of maternal separation with an additional 30 minutes of novelty stress. Relative optical density levels of mRNA expression are expressed in arbitrary units. Data represent mean ± S.E.M. * $P<0.05$ (significant from basal), # $P<0.05$ (significant from separated).

2.4.5.3 Ghrelin (*Figure 2.6.B*)

A main effect of time was measured ($F(1,47)70.51$; $P<0.001$), but not of treatment. Ghrelin levels were twice as high after a first ($P<0.01$), but also after a third period of maternal separation ($P<0.01$), as compared to basal levels. Basal levels were indistinguishable between both treatment groups.

2.4.5.4 c-fos *mRNA expression* (*Figure 2.7, Table 2.2*)

The expression of *c-fos* mRNA in the PVN showed a main effect of time ($F(2,39)43.32$; $P<0.001$). In response to maternal separation *c-fos* mRNA expression increased in the animals separated for the first time ($P<0.05$), whereas it did not respond in the third time separation group. Basal levels were comparable between both treatment groups. Upon exposure to a novel environment for 30 minutes *c-fos* mRNA expression increased to comparable levels in animals separated both for the first and third time ($P<0.05$). *C-fos* mRNA expression in the cortex and in the pPVTh (paraventricular thalamic nucleus) was unaffected by treatment or time.

2.5 Discussion

The present study shows in CD1 mice that the infant mounts a large response of ACTH and corticosterone after 8 hours of separation from the mother. Contrary to our expectations, daily repeated separations of 8 hours readily abolished the pituitary-adrenal response, producing corticosterone levels that were even consistently lower 16 hours after reunion. However, despite the absence of an HPA response to repeated separations, the deprived pup continued to respond to novelty. The finding that the response to multiple separations was reduced below control levels, while stress responsiveness remained enhanced, indicates pronounced different effects on the

neuroendocrine systems of the newborn that regulate basal secretions as opposed to those that require mild stressors.

The results of this study reject the hypothesis that daily separations would lead to an enhanced adrenal corticosterone output. Surprisingly, the HPA axis rapidly desensitised. Moreover, 16 hours after reunion corticosterone levels were consistently lower and this state of reduced corticosterone persisted over the next two days. Recently, there has been an increased awareness of the significance of hypocortisolism, since there is a reduced cortisol secretion observed in children exposed to adverse early life experience [9, 10, 46]. This study is the first demonstration that the same phenomenon also occurs in rodents. It raises the intriguing possibility that a 'normal' glucocorticoid level is required for brain development and that either too low or too high levels may have detrimental effects.

In Experiment I, an 8 hours episode of maternal separation was used to ensure an activation of the pup's HPA axis [17, 31, 39]. When it appeared that after three daily periods of 8 hours maternal separation the endocrine response associated with maternal absence was no longer present, at first a possible shift in time course of the response was investigated (Experiment II). The reasoning was that repeated separations might have facilitated the onset of a transient HPA response to an earlier time point with corticosterone levels returning already to baseline at the 8 hours interval recorded in the first experiment. The time course study demonstrated that there was no earlier response and that the 8 hour interval was appropriate to reliably monitor desensitisation of the ACTH and corticosterone response to repeated separations.

The time course experiment involving 1 hour episodes also showed after the first separation that the initial ACTH elevation occured after 3 hours, whereas corticosterone started to rise only after 6 hours. This indicated that the HPA axis responded slowly to maternal separation and that it took a few hours of ACTH stimulation before the strongly reduced adrenal sensitivity to ACTH during the SHRP was overcome. This observation is in support of previous studies [17, 30, 44].

Experiment II also demonstrated that, if immediately after separation the infants were also exposed to a 30 minutes novelty stressor, a profound ACTH and corticosterone response occured under conditions that the pup's HPA response to repeated separations was abolished. Therefore, repeated maternal separations appear to cause a permanent disruption of the SHRP, as was demonstrated by the sustained responsiveness to novelty in the repeated 8 hours maternal separations paradigm. One might argue that in this respect the pup's HPA axis resembles adult desensitisation to a homotypic rather than a heterotypic stressor. However, the fact that this mechanism already would be in place in the 3-5 days old pup is another demonstration of the remarkable plasticity of the infant's brain. Moreover, as will be pointed out below (see Experiment IV) if repeated separation and novelty exposure are combined the pup's HPA axis desensitises to separation, but not to novelty.

In Experiment III the age dependency of the desensitisation to repeated maternal separations was investigated. The data showed that the endocrine response to maternal separation gradually diminished with each successive separation for both corticosterone and ACTH; *i.e.* the

responses at pnd 4 were lower as compared to pnd 3. The experiment confirmed that basal levels of corticosterone and ACTH at 16 hours after reunion were even lower if compared to non-separated animals. Furthermore, we observed that the endocrine response to a single separation was present at pnds 3, 4 and 5, like previously was shown for pnd 8 [39]. These results indicate that the pups truly desensitise to daily maternal absence and that this desensitisation is not age-specific.

Since it was already shown at pnd 8 that mRNA expression of central HPA axis markers were responsive to 8 hours of maternal absence, several of these markers were examined in our paradigm in order to find clues towards the cause of the observed desensitisation. At pnd 8 CRH mRNA expression in the PVN decreased already after 8 hours of maternal absence [39, 42]. In our paradigm using 3 days old mice CRH mRNA in the PVN only responded to the first period of maternal absence at 16 hours after reunion. Since CRH mRNA expression is corticosterone responsive [4, 33], this downregulation in CRH mRNA expression might be a consequence of the high corticosterone concentrations induced by the first separation period. Concomitantly, the progressive attenuation of the corticosterone response during the second and third separation, respectively, might have been permissive for CRH mRNA expression levels to restore. Alternatively, lower CRH mRNA expression reflects the lower basal ACTH and corticosterone levels at the start of the second separation period. However, the decreased CRHmRNA level recovered after the second separation while this is not the case with the hypocorticism, suggesting that the persistent hypo-responsiveness in the repeatedly separated mouse occurs at the level of the adrenal itself.

Most central HPA markers remained stable during repeated separations, with the exception of the MR mRNA expression in the dentate gyrus (DG) of the hippocampus, which showed a gradual increase. An altered functionality of both receptors has been implicated during 'normal' postnatal development in response to prolonged maternal absence [33, 35, 41, 48]. Already under undisturbed conditions mRNA expression of MR in the DG gradually increased with age [38]. Here, the developmental increase of MR mRNA expression in the DG of the hippocampus seemed facilitated during repeated separations. MR is under positive regulation of CRH in adults, *i.e.* higher CRH levels translate into higher hippocampal MR levels [8]. In support of this reasoning in neonatal CRHr1 limbic brain-specific knockout mice MR mRNA expression is decreased [37]. Though we did not measure CRH levels in limbic regions beyond the PVN, the CRHr1 system presents a good candidate for the facilitated developmental increase in MR mRNA expression in repeatedly separated mice.

The absence of an endocrine response to a third maternal separation and the enhanced response to novelty directly following this third separation seems to indicate that these mouse pups are able to dissociate between maternal absence on the one hand and the stress of a novel environment on the other. To investigate whether we truly observed dissociation between both conditions, we also tested in Experiment IV animals in which each maternal separation was linked to a subsequent novelty exposure for 30 minutes. If pups would habituate to the whole procedure, the response to novelty would no longer be present after "repeated separations with

novelty (SEP+N)". However, for pups treated with this paradigm a novel environment was still stressful at the third exposure.

Interestingly, the ACTH response in the three times separation with three times novelty (3rd SEP+N) group was much less than in animals that were only separated from their morhters for three times (3rd SEP). At the same time, the corticosterone response remained of similar magnitude. Apparently prolonged maternal separation first overcomes the reduced adrenal sensitivity associated with the SHRP [17, 30, 44], whereas further stimulation by daily exposure to a novel environment thereafter enhances sensitivity of the adrenal for ACTH. These data suggest that enhanced adrenal responsiveness to ACTH is the signature of a permanent disruption of the SHRP under conditions of repeated separation combined with novelty stress. Whether other stress factors occurring around the time of maternal separation are implicated in modulation of adrenal sensitivity is not known. It thus seems that repeated maternal separations combined with additional stressors rather than maternal separation *per se* may cause the unwanted daily overexposure of the brain to high glucocorticoid concentrations programming brain and behaviour for later life [5, 22, 51].

The current findings raise the question why the pituitary-adrenal response ceases and becomes hypoactive after repeated daily maternal separations. One mechanism could be related to the pattern of food intake, because feeding is required to maintain hypo-responsiveness of the adrenal to stress [40, 45]. Eight hours of food deprivation decreases circulating glucose and increases ghrelin levels, while administration of a ghrelin antagonist can prevent the separation induced HPA activation [40]. Hence, the next 8 hours could have led to an altered pattern of food intake that is perhaps reflected in the circulating level of these metabolic signals. Experiment V shows that after repeated maternal separations glucose and ghrelin levels still responded similarly as after the first separation event, a finding which is not in support of a role of ghrelin in the desensitisation process. Furthermore, if there were altered patterns of food intake this is not refelected in these metabolic signals.

Plasma leptin levels, relevant in relation to ghrelin [1, 13], were not measured in the pups. Previously, Schmidt *et al.* [40] showed that preventing the maternal separation-induced decrease in circulating leptin levels could not prevent the pituitary-adrenal response to maternal separation, which suggests that leptin is not implicated. High levels of leptin in the neonatal period are, however, required for the formation of projection pathways from the arcuate nucleus to hypothalamic regions that regulate feeding behaviour [1]. Consequently, it is conceivable that repeated maternal separations, which result in episodic metabolic responses, can have a wide range of structural effects with long-term consequences for brain development and functioning [13]. Further experiments are needed, which may include the assessment of ghrelin receptor expression in the arcuate nucleus as well as the role of leptin in structural remodelling of hypothalamic pathways [1].

Another mechanism may be related to the lack of sensory stimulation, since it is known for some time that the activation of central components of the HPA axis of the deprived pup

can be prevented by stroking [45]. Moreover, upon reunion the pup receives excessive maternal care, which also could affect HPA activation the next day. Experiment V demonstrated that after repeated separations the basal *c-fos* mRNA response in the PVN was attenuated, becoming indistinguishable from controls. Yet the PVN *c-fos* response to novelty remained facilitated in the separated animals, which is reminiscent to the response pattern of the HPA axis. Interestingly, in neonate rats handled daily for 15 minutes from pnds 2 to 9, an enhanced response of *c-fos*-immunoreactivity was observed in the (paraventricular thalamic nucleus) pPVTh measured 30 minutes after reunion at pnd 9 as compared to undisturbed controls and pups handled once only at pnd 9 [6]. In the current study *c-fos* mRNA did not respond in pPVTh, a brain region important for relay of sensory information. Also elsewhere in the brain we did not find *c-fos* mRNA changes.

That *c-fos* mRNA in the hypothalamus displays a corticosterone-like response pattern to repeated separations suggests that a central mechanism is involved. It may well be that even the 3-5 days old infant can already predict after one experience that the mother will return in 8 hours. The infant thus rapidly adapts or habituates to maternal absence, but the HPA axis stays on alert and can be activated by stressors. Recent evidence suggests indeed that learning can occur in neonatal rats [25, 26, 43]. In these studies it was found that the first week of life attachment of the infant through olfactory cues from the mother depends on a locus coeruleus – olfactory pathway. In the first postnatal week infant rats rapidly learn maternal odor to support attachment behaviour and to suppress odor aversions during that time. It is only in the absence of the mother that aversive odors start to activate the amygdala circuit that governs fear-motivated avoidance. This switch from maternal attraction to avoidance is facilitated by corticosterone and can be blocked by a glucocorticoid antagonist. It is conceivable that such a corticosterone-dependent mechanism may form the basis for adapting to the transient nature of maternal absence, since the infant is still capable to mount a corticosterone response upon exposure to novelty.

The implications of our findings are very interesting. It would imply that the outcome of the repeated maternal separations would be, besides gender and strain, not only dependent upon the duration of the separation and the maternal care received by the pup upon reunion, but also on the pup's stressful experiences during the separation procedure. That maternal care is not the sole factor determining epigenetic programming and outcome was recently demonstrated in the elegant studies of Macri and Würbel [20, 21]. Therefore, standardisation in protocols used for study of the outcome of maternal separations is required, since the current data show a different outcome if the pup is removed, the dam, or both.

In conclusion, the current data demonstrate the amazing capacity of the newborn rodent to readily adapt to the absence of the mother and present a striking example of plasticity, since the infants remain responsive to novelty and seem to be marked by a too low circulating amount of corticosterone. The finding may be helpful to design rational experiments to understand the mechanism underlying the lasting effects of early adversity on brain and behaviour.

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